AD-8027

FINAL REPORT

Report #8

January 1948-June 1952

For the Office of Naval Research
Microbiology Branch

# PHYSIOLOGY OF THE WOOD-ROTTING FUNGI

Contract N6-onr-248, T. O. II

Designation Number NR 132-159


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September, 1952

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## PREFACE

The present report is the final one on this project. It comprises the material in the following technical reports, which material has been corrected where needed, expanded, brought up to date, and put together so that all of the studies on a given point are a unit. A review of the literature has been added.

Summary Technical Report, #1, January - December, 1948
" " " , #2, January - December, 1949
Semi-annual Progress Report, #3, January - June, 1950
" " " " , #4, July - December, 1950
" " " " , #5, January - June, 1951
" " " " , #6, June - December, 1951
" " " " , #7, January - June, 1952

## INTRODUCTION

Some 2000 species of so-called "wood rots" are known, of which perhaps 200 to 300 are commonly involved in wood deterioration on a practical scale. They typically attack woody materials—either as living or dead trees, structural timbers or woody detritus in nature. The wood rots are the chief fungi, if not the only ones, that can attack the cellulose-lignin complex which constitutes wood. In nature, the useful activities of these organisms are primarily those of getting rid of dead organic matter. The undesirable activites of the organisms are, of course, their effects on structural timbers. The very fact that in the degradation of cellulosic materials they produce other chemical compounds, suggests that the activitives of the wood rots can perhaps be turned into economically useful channels.

Before intelligent control can be attained of either type of activity of the wood rots-- prevention of wood decay, or utilization of waste cellulosic materials by fermentation--it is necessary to know more about the physiology of the organisms. Very little work has been done on any aspect of the fundamental biology and chemistry of even a few of these fungi. Much less has an attempt been made to investigate thoroughly and systematically the basic functioning of the organisms. The present investigations are a start towards this objective.

## REVIEW OF LITERATURE
(See Bibliography A)

### A. GROWTH, NUTRITION, METABOLIC PRODUCTS OF WOOD ROTS.

The first intensive study of the fungi causing wood decay was undertaken in 1930 by Falck (23). More recent reviews of the destruction of cellulose and cellulosic materials by microorganisms include those by Boswell (9), Burkholder and Siu (14), Cartwright and Findlay (18), Hartley (33), and Row (75). Harris and Johnson (32) discussed the microbiological utilisation and disposal of wood-processing wastes.

Natural decomposition of wood is the result of successive attack by different types of organisms. The initial breakdown is caused by the *Phycomyces*, which utilize the water-soluble material, followed by bacteria and fungi which attack starch, cellulose, and hemicelluloses. The most resistant portions, namely, the lignified tissues, are broken down by bacteria and "higher" fungi (97). The fungi causing wood decay are mainly Basidiomycetes and belong to the families Theleophoraceae, Hydnaceae, Polyporaceae, and Agaricaceae (18). The type of fungal decay depends not only upon the species of fungus, but also upon the kind of wood and the particular portion of the wood being attacked.

The sequence of species in decay of conifers under natural and controlled conditions has been studied by Findlay (25) and Cartwright and Findlay (18). The sapwood is first attacked by sap-stain fungi, then by sap-rotting fungi. The white-rotting species of *Polyporus* are responsible for decay of the heartwood. Using the block culture method, *Polyporus vaillantii* decayed 55 per cent (by weight) of the block, *Polyporus versicolor* completely destroyed it, and *Coniophora cerebella*, *Merulius lacrymans*, and *Lenzites trabea* degraded up to 70 per cent of the block. The brown-rots were found to preferentially attack conifers, while the white-rots generally attacked only the hard woods. Two species were rarely isolated from the same region of the wood, presumably because of the different nutritional and pH requirements. Experimentally, greater decomposition of wood resulted from attack by mixed cultures of fungi (78), or by the presence of saprophytic bacteria (78, 94). The rate of natural decay then, would seem to be dependent upon the availability and concentration of food, the presence of various inhibitory substances, pH, and other growth conditions.

Conditions for growth of wood-rotting fungi

The wood-destroying fungi are, in general, mesophilic and may be separated into three groups according to their temperature requirements. The majority grow optimally within the range of 24-30°C, while others prefer a higher temperature (30-34°C), and a few require a lower temperature (20-24°C). Several investigators have studied the optimum temperatures for growth on either agar (18,86) or wood blocks (16), and also the temperatures lethal to the organisms (16, 61). Humphrey and Siggers (37) surveyed the temperatures for optimal growth of 64 species of wood-rots grown on two different media. The optimum temperature was determined by measuring the growth increment on Petri dish cultures after one or two weeks incubation. The optimum temperatures, temperature range, and maximum inhibitory temperatures were tabulated.

The wood-rots, in general, prefer an acid medium for growth. The optimum for some species may range as low as pH 3. During growth the pH of the culture fluid or substrate usually becomes lower due to the production of organic acids (85,94,98,102,103). The brown-rots produce more acid than the white-rotting species (3). Thaysen and Bunker (94) found that although many fungi prefer an initial acid reaction, the presence of alkaline salts, for example, sodium carbonate, stimulates the rate of decay of wood, presumably by neutralizing the organic acids produced by the fungi during growth. The increased decomposition of wood in the presence of saprophytic bacteria has been postulated to be due to a similar neutralization of toxic metabolic products. The moisture requirements of the wood-rotting fungi are variable. Most of the studies relate moisture requirements to the rate of decomposition of wood (80,98).

The wood-rots have been successfully grown using the surface culture technique on both solid and liquid substrates. Recently, however, the shake culture method, originally introduced by Kluyver and Perquin (50), has replaced other methods, especially in physiological studies of fungi in general (42,43,44,45,46,60). Foster (28) gives a comparative physiology of surface vs. submerged growth. He states that surface culture is entirely inadequate because it represents only the over-all result of the metabolic processes of an heterogeneous mixture of physiological systems. Submerged culture, however, provides physiologically homogeneous fungal material since all cells are uniformly exposed to the environmental factors, both physical and chemical, during the growth period. Certain chemical changes in submerged fermentations were reviewed by Koffler <u>et al</u> (51), and the effect of shaking on oxygen diffusion was studied by Starks and Koffler (88). Burkholder and Sinnott (13) investigated the morphology of 150 species of fungi, including some Basidiomycetes, in liquid shake culture. The mycelial masses were characterized as being either globous or irregular, hirsute or smooth, and either hollow or solid in texture. The type of intermycelial fluid was also studied. They also discussed the effects of environmental conditions on these characteristics.

In order to use the submerged culture technique for quantitative measurements of growth, conditions necessarily must be constant. The rate and total amount of growth depend upon the size and type of inoculum (28). It has been found that a closer check of replicate cultures and a shorter lag period in initiation of growth are attained if fungal mycelium is fragmented (a Waring blendor is usually used) (21, 77). Time of blending, washing and suspending of fragments, and amount of suspension used for the inoculum are important factors. The effect of size of inoculum was investigated by Kitay and Snell (47). The organisms may store (some may adsorb) several times their requirements of vitamins when grown on a vitamin-rich medium. The amount of inoculum transferred to a vitamin-deficient medium, therefore, is critical, and repeated transfer is necessary to eliminate carry-over of vitamins.

Nutrition of the wood-rotting fungi.

The nutritional aspects of the wood-destroying fungi have not been studied extensively, and until recently a chemically-defined medium for growth had not been developed. Excellent growth has been obtained in a variety of non-synthetic liquid media such as bran or malt extract with supplements of yeast, nitrogen compounds, etc. (104). Various synthetic or semi-synthetic media have been developed for growing basidiomycetous fungi (1,36,39,55,62,69,89,93) and related organisms (10,30,83,87). Most of the media, however, contained certain organic supplements the exact nature of which is unknown. Studies to determine nutritional requirements, especially vitamins, would have little significance if such media were employed. Perlman (70) has developed a completely synthetic medium for culturing *Polyporus anceps*, which contains glucose, inorganic nitrogen, inorganic salts, trace elements, and thiamine.

Nitrogen utilization was studied by La Fuze (55) who found that growth decreased as the nitrogen constituent became simpler; that is, proteins, casein, gliadin, gelatine, peptone, amino acids, and inorganic nitrogen sources, respectively, resulted in decreasing amounts of growth. According to his studies, sodium nitrate and urea were non-nutritive, while proteins high in tryptophane, ammonia, or glutamic acid favored growth. Washburn and Niven (99) suggested that several interrelationships existed in amino acid assimilation. They found that several amino acids were deaminated and then the carbon chains were aminated to form glutamine units. Knight (54) found that certain test fungi contained L-amino acid oxidase and probably only the L-form could be metabolized.

Tally and Blank (93) studied certain factors influencing utilization of inorganic nitrogen. They included the source of carbon, the presence of heavy metals, and most particularly the vitamins added. The latter were introduced in the form of yeast supplements so that error due to the introduction of contaminating substances was not eliminated. Brickson et al (12) demonstrated antagonistic effects upon growth by an improper balance of the concentrations of amino acids and proposed that inhibition of growth by a particular amino acid was probably due to prevention of amidation of the glutamic acid. In a recent study, Stephens and Hinshelwood (91) found that optimum rate of growth was not dependent upon trace elements, but on the addition of a full complement of amino acids. Theoretical kinetic relationships were given as supporting evidence. Care must be taken in evaluating these experiments since it was found that autoclaving amino acids, particularly in the presence of sugar, caused partial or complete inactivation of several amino acids (22).

Various nutritional factors have an effect upon the rate of decay of wood. The presence of bacteria increases the rate of decomposition of complex plant materials (97) and wood (78,94). Findlay (24) found the effect of nitrogen content to be variable. Schultz and Kaufert (80) found that asparagine increased the rate of decay of pine by Polyporus versicolor and Lenzites trabea, while ammonium nitrate had no effect. In another study, Kaufert and Behr (47), using wood blocks, found that the rate of decay of several woods was not affected by urea, ammonium sulfate, or ammonium phosphate in small quantities, but large quantities decreased the rate. Asparagine and peptone, in small quantities, increased decay of Southern pine and red oak but had no effect upon other woods. Hungate (38) determined the nitrogen content of various woods before and after attack by fungi and found that the rate of decay was proportional to the initial nitrogen content of the wood. Schmitz and Kaufert (81), and Findlay (26), found that addition of dextrose or dextrose plus asparagine increased the rate of decay of Norway pine by Lenzites trabea, but decreased the rate of breakdown by Lentinus lepideus. Utilizable carbon sources included starch, maltose, dextrins, and glucose (55).

Several investigators have shown that added nutrilites (especially thiamine) augment the growth of the wood-rotting fungi. Very little work has been done to relate vitamin nutrition and the rate of decay of wood. In this connection, Burkholder and Snow (15) determined, by fungus assay, that thiamine was present in wood, especially in leaves and bark. Noecker (62) found that thiamine was an absolute requirement for growth of four species of wood-rots and that biotin, in some cases, stimulated growth. Perlman (70), using a synthetic medium, found that utilization of glucose by Polyporus anceps depended upon the concentration of thiamine; that is, increased amounts of thiamine resulted in increased utilization of glucose, which in turn produced a large quantity of mycelium per unit volume of medium. Other growth factors, in addition to thiamine, had no effect upon growth. The whole thiamine molecule was required for the growth of this organism. It has been found that the activity of constituent parts of thiamine, namely, thiazole and pyrimidine, varied not only with the species of fungus, but also with the different strains (11,82). Leonian and Lilly (57,58) found that the requirements for the thiamine molecule or its components varied with the source of nitrogen. The importance of other nutrilites in culturing various fungi has been reviewed by several workers (29,52,53,59,95). Kögl (52) found that biotin, thiamine, and l-inositol increased the growth of several species of mushrooms. Adenine, guanine, and/or uracil, although not essential, stimulated growth.

Several interesting relationships between nutrilites and other metabolites have been reported. It was found by Carlson et al (17) that biotin was essential for monosaccharide utilization but was not required when disaccharides were the carbon source. Knight (53) found that thiamine requirement was altered by the presence of either niacin or biotin. The latter has been implicated in bicarbonate utilization (56) and the synthesis of aspartic acid (92) by fungi.

Robbins and Kavanagh (73) obtained luxuriant growth of several thiamine-requiring fungi on a synthetic agar medium. Further investigation showed that agar contained a higher concentration of thiamine than that required for growth of the organisms. Ryan, Beadle, and Tatum (76), in connection with the development of the tube method of measuring growth rate of the organism Neurospora, found that biotin was adsorbed onto the glass in relatively high concentrations. Since the amounts of nutrilites required by microorganisms is extremely small, care must be taken to eliminate all sources of contamination before absolute requirements are established.

Study of the heavy-metal nutrition of fungi was accelerated when the importance of trace elements in commercial fermentations was realized (27, 28,69,70,90). Jarvis and Johnson (41), and Shu and Johnson (84), respectively, studied the effect of heavy metals on penicillin production and on citric acid production. These investigations suggest a good approach to similar studies with other fungi. Schmitz (79) found that the rate of decay of Douglas fir sawdust by Lenzites saepiaria was increased by sodium carbonate, bicarbonate, sulfate, and chloride and related this to the effect of alkaline soils on the rate of decay of wood in contact with them. Kaufert and Schmitz (48) studied the effect of arsenic, zinc, and copper on the rate of decomposition of various woods by fungi. Sinden et al (85) related the mineral nutrition of certain wood-rotting fungi to their cellulolytic activity.

Certain metabolic products of wood-rots

The major products of aerobic decomposition of carbohydrate by fungi are carbon dioxide and cell substance. Various intermediates and end products have been isolated and identified from the metabolic fluid and/or the mycelium of the wood-rotting fungi. They include several organic acids, aldehydes, aromatic substances, products resulting in coloration of the substrates, etc. Curtin (19) proposed the hypothesis that wood degradation was due in part to the acids produced by fungi. Experimentally, he showed acid production in malt agar and in wood sticks. Birkinshaw et al (3) isolated formic, acetic, oxalic, and citric acids from cultures of Coniophora cerebella on Scots pine. All acids, except the latter, were isolated from wood, so that citric acid was probably the only true metabolic product. Acetic and succinic acids have also been isolated as two products in the breakdown of glucose. Perlman (70) found that glucose dissimilation by Polyporus anceps resulted in the production of ethanol and acetic and oxalic acids. More mycelium was obtained from starch metabolism than from the utilization of an equal quantity of glucose. A similar study was carried out by Boswell (8) with Merulius lacrymans.

Methyl-p-methoxycinnamate was identified as a metabolic product of Lentinus lepideus grown on either glucose or xylose. Methyl cinnamate and an ester of anisic acid were products of this organism when cultured on Scots pine (2,66). Birkinshaw et al (4) identified D-threitol (l-erythritol) as a product of Armillaria mellea. These products are responsible for the characteristic odor and/or coloration of cultures of the two organisms. Hamada (31) studied the secretory reactions of 9 strains of Armillaria mellea, including luminescence, browning of substrate, guttation from aerial mycelium, color of fluid of guttation, and formation of calcium oxalate crystals. He found that these reactions were dependent upon the nitrogen/carbon ratio of the medium, except the browning of the substrate, which resulted from the oxidation of peptone and so varied only with the nitrogen concentration.

Other metabolic products of the wood-rots include various enzymes, antibiotic substances, and pigments. Nord et al (63,64,65,67) have extensively studied the enzymes involved in the action on glucose, xylose, raffinose and cellulose, and the mechanism of wood decay. Vitucci et al (96) studied the dehydrogenases of several species of Merulius and Fomes annosus. Bose and Sarkar (7), and Bose (5), also studied the cellulolytic enzymes of the wood-rots. Considerable work has been done on the production of antibiotics by wood-rots (6,40,72,74,100,101). Hervey (34) screened 500 Basidiomycetes for their antibacterial activity. The percentage of active organisms was found to be relatively small. Pigment production by a wood-rot, Lenzites trabea, has been studied (20).

REVIEW OF LITERATURE
(See Bibliography B)

B. CELLULOLYTIC ACTIVITY OF WOOD-ROTS

NATURE OF CELLULOSIC MATERIALS

"True" Cellulose

Pure cellulose is rarely found in nature. Rather, it occurs most frequently in intimate association with other materials. Since these latter substances often affect the reactions of cellulose, an attempt will first be made to elucidate the nature of the substrate which may be attacked by cellulolytic and associated enzymes.

The seed hairs of many plants, such as cotton, are composed of nearly 100 per cent cellulose, free from other substances, and are the purest form in which it occurs in nature. The empirical formula $(C_6H_{10}O_5)_n$ indicates the fundamental structure of cellulose to be a hexose anhydride. Complete hydrolysis of cellulose produces a nearly quantitative yield of glucose; the qualitative relationship between the two substances has been known for more than a century and a half. Data supporting the classical theory of cellulose structure are exhaustively discussed elsewhere (24, 27, 44). Here it may be pointed out merely that cellulose is made up, basically, of B-D-glucopyranose units joined by 1, 4-glycosidic bonds. The cellulose fiber is composed of chains of these residues, varying from extremely long chains containing hundreds of glucopyranose units, down to a few cellobiose or even glucose units. The generally assumed hydrogen bonds between parallel chains partially account for the lateral union of the chains. Further, the primary chain molecules are joined by a linkage involving their terminal open chain glucose units united by a cellobiose molecule (24).

X-ray data support the B-D-glucopyranose structure theory and, moreover, demonstrate the crystalline nature of cellulose. The long-chain molecules are organized into parallel bundles by the previously mentioned lateral cohesive forces. The bundles so formed assume a crystalline structure called a micelle. Separating the crystallites are areas which do not show the crystalline pattern, presumably because the arrangement of the molecules is less parallel. The cellulose fiber is, then, a biphasic system; the phase, crystalline or amorphous, depends upon the physical organization of the constituent chains. It is thought that the less organized spaces are more easily penetrated than the micelles and that those reactions involving penetration of the cellulose fiber are accounted for by this structural difference (24).

Wood Cellulose And Other Wood Constituents

The cellulose of wood, which comprises a large portion of the woody structure, is thought to be identical with the "true" cellulose previously discussed. Several sets of data support this contention. Wise (43), in a discussion of the work of Heuser and Boedeker, as well as that of several other authors, presents evidence strongly in favor of the contention that wood cellulose is identical with other celluloses. More recently, in their review, Nord and Vitucci (24) have concluded that this opinion is justified.

The natural breakdown of wood cellulose, however, may be markedly influenced by other wood constituents. For example, wood-rot metabolism is critically affected by the extractive content of wood. In the aggregate, or possibly alone, volatile oils and acids, tannins and other extraneous substances are inhibitory to wood-decomposing fungi (16, 34, 39). Although extractives may be stimulatory in extreme dilution (39), higher concentrations are fungistatic, more is lethal.

The other major component of wood, lignin, which is found in intimate association with cellulose, has a definite effect on cellulose destruction. The protection against microbial destruction afforded by lignification is amply illustrated by a comparison of the rates of decay of non-lignified or slightly-lignified materials, such as cotton or leaves, with those of more highly lignified substances such as wood. With an increase in lignification there has been shown to be an increase in resistance to microbial attack.

The molecular structure of lignin, while not completely established, consists basically of phenyl propane. The molecular weight is, at present, thought to be about 840, with an empirical formula $C_{46}H_{48}O_{15}$. In addition to the basic skeletal structure, several hydroxyl and methoxyl groups have been found. There is evidence, too, of one carbonyl group and one enolic group. Brauns' formulation, which indicates a keto-enol isomerism, best agrees with reviewed data (24).

The manner in which wood cellulose is bound to lignin is a matter of prime importance, since resistance of the cellulose-lignin complex to enzymic attack may well be due to the nature of the union. The kind of union between lignin and cellulose is highly controversial.

Consideration of the cellulose macromolecular structure leads to the conclusion that the introduction of a unit as large as lignin into this structure would result in serious distortion, probably to the point of molecular disruption (27). This militates against the concept of a strong chemical union. Also, as previously noted, wood cellulose and other celluloses are probably identical. This implies that a strong chemical linkage with lignin is highly improbable, although it will admit the possibility of an occasional chemical bond between lignin and the surface units of the cellulose macromolecule.

Nord and Vitucci (24) have reviewed the evidence supporting both the chemical bond and physical union concepts. They conclude that experimental data are in favor of the non-existence of a chemical linkage, while the evidence for at least some of the lignin being in a free state is incontrovertible. The effect of particle size on cellulose degradation offers further corroborative data, finely-ground wood particles being more readily attacked than large particles or intact wood.

MICROBIAL ACTION ON SUBSTITUTED CELLULOSES

In addition to their well-known ability to attack natural cellulosic materials, microorganisms may exhibit hydrolytic activity on cellulose derivatives. Several substituted celluloses have been examined for their ability to resist enzymatic action. With the increasing use of cellulose derivatives for myriad commercial products, this aspect of the cellulose deterioration problem assumes an increasing economic importance.

That cellulose can be rendered more resistant to microbial attack by chemical substitution has been known for some time. One of the earlier (1921) published reports was that of Doree (9), who noted the resistance of cellulose acetate to the microbial action of sea water. This property of cellulose acetate has been confirmed by several investigators. Recent publications (5, 8, 17, 36) dealing with the degradation of substituted cellulose indicate some of the applications of such studies. In the studies cited several cellulolytic microorganisms were investigated, using various substituted celluloses as substrata. The growth response of the organisms was taken as a criterion of utilization. Results showed several natural fibers to be vulnerable, only small differences in vulnerability being noted. Substituted celluloses, on the other hand, showed a wide variation in immunity to attack. Cellulose triacetates were completely resistant to attack, as was more highly substituted cyanoethylated cellulose. Low substituted cyanoethyl cellulose and methyl cellulose appeared to be attacked, however.

Darby (8) has tested the resistance of several substituted celluloses to the action of *Myrothecium verrucaria*, a highly active cellulolytic organism. He found the following to be completely immune to attack: ethyl cellulose, (45-49.5% ethyoxy), acetyl cellulose (22% acetyl D.S., degree of substitution, = 1), triacetyl cellulose, acetyl butyryl cellulose (16% butyryl), acetyl hydrogen phthalyl cellulose, acetyl stearyl cellulose, tosyl cellulose (D.S. = 1.29-2.01), idotosyl cellulose (D.S. = 1.29-2.01 with 0.60 to 0.86 tosyl groups converted to idotosyl), cyanoethyl cellulose (D.S. = 1.02-2.90), and the mercury salts of carboxymethyl cellulose.

Partial resistance was found in the following: mercerized cotton, regenerated cellulose (as cellophane)*, methyl cellulose, carboxymethyl cellulose as free acids, the sodium salts of carboxymethyl cellulose of low, medium and high viscosity, aluminum salts of carboxymethyl cellulose, and oxidized cellulose.

No significant differences in susceptibility to attack were found among filter paper, dewaxed cotton sliver and extracted natural linen.

ENZYMATIC DEGRADATION OF CELLULOSE

Since cellulose is the most abundant organic compound in nature, it is not surprising that cellulose-decomposing activity is widely found among microorganisms. Cellulolytic organisms may be found among the following groups: aerobic and anaerobic bacteria, some actinomycetes, filamentous and higher fungi, protozoa, insects and invertebrate animals (41). Some of these organisms are highly specialized, requir-

* Darby, (loc. cit.) This is at variance with the report of Burkholder and Siu (5), and of Saunders, Siu and Genest (32).

ing cellulose as the only carbon source; others, not so specialized, may utilize a number of carbon sources, cellulose among them.

Some interesting interrelationships exist between cellulolytic organisms and non-cellulolytic species. One of the more often used illustrations of symbiosis is that existing between the ruminant and microorganisms inhabiting its digestive tract. The latter organisms produce short-chain acids from cellulose in the food, which acids, unlike cellulose, are available for metabolism by the host. This type of interdependence among organisms is not limited to the example cited, as other microorganisms, including protozoa, act in a similar manner on behalf of their hosts, which in turn protect and supply nutrients to the microbes.

Deterioration of wood is due primarily to fungi. It is not, however, in most instances carried to completion by any single type of organism. Rather, wood is attacked initially by several non-cellulolytic types of fungi which utilize the sugars and starches but not the wood substance. When the nutrients available to these organisms are diminished the true wood-destroying fungi invade, causing final destruction of the cellulose and lignin (41). These latter organisms may also utilize carbohydrates other than cellulose.

Nature of the Cellulolytic Reaction

Initial attack on the cellulose macromolecule is most likely a hydrolytic one. The classical view of cellulose hydrolysis, and one which is currently widely accepted is:

$$\text{cellulose} \xrightarrow{\text{cellulase}} \text{cellobiose} \xrightarrow{\text{cellobiase}} \text{glucose}$$

Pringsheim (28) showed both glucose and cellobiose to be present in the hydrolytic products of cellulose acted upon by thermophilic bacteria. However, the possibility of the release of intracellular ensymes as a result of lysis of cells under conditions of the experiment is pointed out by Levinson and Reese (17). Further, Kalnins (15), cited by Norman and Fuller (25), was unable to detect cellobiose in the fermentation of cellulose by mesophilic organisms.

The work of Grassman *et al*. (10), cited by Levinson and Reese (17), in which two ensymes were separated from a dialysed solution, requires a different representation of the cellulose-to-glucose hydrolysis. The ensymes consisted of a "cellulase" capable of hydrolysing chains having as few as 6 anhydroglucose units, and a B-glucosidase capable of hydrolysing chains two to six units in length but which had little action on more highly polymerised chains. The reactions may be represented as follows:

$$\text{cellulose} \xrightarrow[\text{cellulase}]{\text{1,4 B-polysaccharase}} \underset{(n=2\text{-}6)}{(C_6H_{15}O_5)_n} \xrightarrow[\text{cellobiase}]{\text{1,4 B-oligosaccharase}} \text{glucose}$$

A recent publication by Reese et al. (31) represents the reaction as follows:

$$\text{native cellulose} \xrightarrow{C_1} \text{short linear polyanhydroglucose chain} \xrightarrow{C_x} \text{soluble small molecules capable of diffusion into the cell}$$

The $C_1$ step occurs preliminary to hydrolysis of the straight chain by $C_x$. The authors used carboxymethyl cellulose (CMC) as a substrate for the detection of $C_x$ activity and, in this connection, made the following statement, "We are restricting the use of the term $C_x$ to the enzyme capable of hydrolysing the 1,4 B-glucosidic linkage as found in cellulose and as measured by the amount of reducing sugars obtained by hydrolysis of CMC." Further, "The term cellulolytic is used in the broader sense indicating the ability to attack or hydrolyze native cellulose (cotton and filter paper). $C_x$ alone is unable to hydrolyze these materials." The enzyme $C_x$ was able to hydrolyze starch, pectic acid, alginic acid and bacterial dextran. It was found in all cellulolytic organisms tested and in some non-cellulolytic ones.

As previously mentioned, there is a relationship between the molecular structure and susceptibility to enzyme attack. Reese et al. (31) state that in experiments using substituted celluloses, they found that a single substituent on each anhydroglucose unit rendered the derivative immune to attack. Moreover, as this D.S. (degree of substitution) value decreases there is a decreased resistance to enzymic action. While the degree of substitution is a determining factor in hydrolysis rate it is indicated that D.P. (degree of polymerisation) is not.

Greathouse (11) prepared a series of polyhomologous hydrocelluloses ranging in D.P. from 1675 to 120. His data indicated no significant difference in attack by Myrothecium verrucaria on the various members of the series. This is in accordance with the findings of Siu et al. (36), and with those of Walseth (42), who found no significant relationship between the ease of enzymic hydrolysis of cellulose and its degree of polymerisation. Greathouse considers that enzymic attack is random along the cellulose chain.

This is at variance with Clayson's end-wise attack (7). Since a decrease in D.P. would increase the number of terminal glucose units available, an end-wise attack would give greater reactivity as the D.P. decreases.

Using commercial enzyme preparations as a source of cellulase, and cotton linters, swelled or dispersed with phosphoric acid, as substrates, Walseth (loc. cit.) has found that, as the reaction progresses, there is a marked decrease in reactivity of the undissolved cellulose. Decreased moisture-regain values for the undissolved residue indicate the amorphous region of the cellulose to be attacked most rapidly, which could account for the reactivity decrease, since the extent of attack would be limited by a decrease in relative amounts of amorphous cellulose. A comparison of enzymic hydrolysis with acid hydrolysis indicated that the large enzyme molecule cannot penetrate the inter-crystalline spaces of the cellulose macromolecule, with the result that only those chains which can be contacted are depolymerized. The residue, then, was highly polymerized, though much of the sample was dissolved. The hydronium ion, on the other hand, could penetrate the cellulose structure and hydrolyze the more interior chains with the result that hydrolysis, to the extent of appreciable weight loss, results in a low-polymer residue.

Cellulose treated with phosphoric acid prior to enzyme action, results in a "high reactivity cellulose" due to a swelling of the cellulose structure, thus facilitating enzyme penetration. Reactivity increases as the length of treatment time increases with the resultant extension of the crystal lattice (42).

THE WOOD-ROTS AS CELLULOLYTIC ORGANISMS

As previously pointed out, the wood-rots play a leading role in the ultimate destruction of woody materials. Numerous organisms, including many fungi, bacteria, protozoa and invertebrates, are possessed of enzyme systems capable of degrading naturally-occuring cellulosic material. The wood-rots, however, are unique among cellulose destroyers in that they are able to enzymatically degrade lignified material. Decomposition of ligno-cellulose, according to Cartwright and Findlay, (6) is restricted to certain Basidiomycetes and a few Ascomycetes. In view of this unique ability, it is somewhat surprising that the enzyme complex of wood-rots has been the subject of relatively little investigation.

There are two principal types of wood decay brought about by wood-destroying fungi. In either type cellulose is degraded; the white rots also degrade lignin, while the brown rots do not attack this latter substance.

Zeller (45) was among the first to investigate the wood-rots for enzyme content. He found a cellulase, among several other enzymes, to be present in Lenzites saepiaria. The vegetative mycelium contained greater amounts of enzyme than the sporophore tissue. Later, Schmitz and Zeller (35) reported a similar group of enzymes occuring in Armillaria mellea, Daedalea confragosa and Polyporus lucidus. Bose and Sarker (4) made an important contribution to the study of wood-rot physiology when they found, in eight species of Polyporaceae, the extracellular enzymes in general to be present in greater amounts than the corresponding intracellular enzymes. The extracellular nature of many fungal enzymes was thus established, pointing out the inadequacy of some earlier experimental work in which, for the most part, mycelial extracts, and inferior enzyme source, were used. Confirming the earlier work of Zeller (45), Bose and Sarker (4) found the vegetative mycelium superior, as a source of enzymes, to fruiting or about-to-fruit structures.

Ploetz (28), using press juice from Merulius lacrymans, precipitated the enzymes with alcohol-ether. He found cellulose to be feebly attacked, and then only by preparations from older cultures. Lichenin and cellobiose, however, were vigorously attacked.

In his review, Bose (3) points out that only about two dozen wood-rots have been studied with regard to their enzyme activity. More recently the problem of cellulose dissimilation has been studied by an investigation of the end products of wood-rot metabolism. Birkinshaw _et al_. (2) found _Coniophora cerebella_ to produce acetic, formic, citric and oxalic acids. Oxalic acid has been demonstrated as a metabolic product of _Poria vaillantii_ (30), and _Merulius lacrymans_. _Lentinus lepideus_, acting on wood, gives rise to methyl-P-methoxycinnamate as a metabolic product (1). Since this product is also produced by the fungus acting on glucose (24), it appears to corroborate the theory that a preliminary step in wood degradation is a hydrolytic action in which glucose is produced.

In a series of papers on the biochemistry of wood-rotting fungi, Nord _et al_. (18, 19, 20, 21, 22, 23, 24, 40), have presented considerable evidence of the fate of cellulose utilized by wood-rots. They followed the stepwise dissimilation of glucose to various end products. Glucose breakdown, in many cases, is principally to oxalic acid. Also produced, in greater or lesser amounts, are ethyl alcohol, acetic acid, acetaldehyde and succinic acid.

Oxalic acid, in those instances where it is produced, apparently results from the oxidation of acetic acid, which may be converted to oxalic acid by either of two metabolic routes (24).

Further work, in which purified cellulose, and in some cases wood, were used as substrates showed the same metabolic products to be elaborated as when glucose was used. From this, according to the authors, it would be justified to postulate a mechanism for cellulose degradation by a consideration of the phase sequence of glucose dissimilation. The theory of preliminary hydrolysis, previously mentioned, is further strengthened.

Nord <u>et al</u>. also made an examination of residual cellulose and showed an increase in reducing power as oxalic acid increased, indicating the organism (<u>Coniophora cerebella</u>) split the 1,4 glycosidic bond, giving rise to new reducing groups. This would overshadow that possible action which would split hemiacetal linkages with its resultant diminution of reducing power.

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## GENERAL AND SPECIFIC OBJECTIVES

### General Objectives

This research has as its broad objective a fundamental study of the nutrition and physiology of the wood-rotting fungi. These organisms - - classified as Basidiomycetes - - include the so-called "brown rots" and the "white rots" which primarily attack, respectively, the cellulose and the lignin of the cellulose-lignin complex of wood. It is hoped that these studies may contribute to the practical problems of the prevention of wood decay and of the fermentative utilization of carbohydrate materials (including waste cellulose), as well as extendingfundamental scientific knowledge of the organisms.

### Specific Objectives

More specifically, this investigation involves (1), a systematic study of the nutritional requirements of representative wood-rotting fungi under controlled conditions of artificial culture; and (2), study of various aspects of the physiology of selected organisms. Some 43 species of wood rots - - representative of different types involved in the decay of wood -- are presently under investigation.

Study of the nutritional requirements (1) involves, among other things, development of chemically-defined (synthetic) culture media for growth. This includes the qualitative needs of all the organisms for "trace" elements and inorganic salts, for nitrogen compounds and carbon compounds, and for vitamins or other nutrilites, and the quantitative characterization of these nutrients for the optimal growth of the organisms.

Study of the physiology (2) includes investigation of the cellulolytic enzymes, which are responsible for the primary breakdown of wood;

utilization of various carbohydrates by the organisms; determination of the end-products of the fermentation of carbon compounds, including waste cellulosic materials; study of oxidation-reduction changes in culture and their relation to growth and fermentation; pH and temperature optima, and related problems.

The above studies of nutrition and physiology are fundamental to a rational approach to the control of wood decay and to the practical applications of the organisms in the fermentation of carbohydrate materials to economically valuable products.

## CATEGORIES OF SPECIFIC STUDIES MADE

1. Growth of wood rots in non-synthetic culture media
2. Development of purely synthetic (chemically-defined) media for growth and nutrition studies
3. Utilization of different forms of organic and inorganic nitrogen
4. Utilization of different carbon compounds
5. Growth curves of the wood rots
6. Vitamin requirements, substitutions, components and synthesis
7. Optimum temperature for growth
8. Optimum pH for growth
9. Oxidation-reduction potential (Eh) in relation to growth
10. Development of synthetic media optimal for growth
11. Separation and concentration of cellulolytic enzymes.
12. Development of "cellulase assay tube" for rapid determination of cellulase activity.
13. Production of organic acids by wood-rotting fungi
14. Pigment production
15. Production of fungal polysaccharides
16. Method for determining cellulose breakdown in sawdust
17. Large-scale growth of wood rots in aerated liquid culture
18. Fermentation of sawdust, bark, and other complex carbohydrate materials
19. Respiration studies

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## LIST OF ORGANISMS USED

| Name | Culture # | Source |
|---|---|---|
| Brown rots-- | | |
| Coniophora cerebella | # 2 | Brazil** |
| Daedalea quercina | FP 57076-S | U.S.D.A.* |
| Fomes meliae | 50336-R | " |
| Fomes officinalis | F 1276 | " |
| Fomes roseus | Snell 11 | " |
| Fomes subroseus | Snell 20 | " |
| Hydnum pulcherrimum | 81027-R | " |
| Hymenochaete sallei | # 3 | Brazil** |
| Lentinus lepideus | 534 | U.S.D.A.* |
| Lenzites saepiaria | 537 | " |
| Lenzites striata | # 1 | Brazil** |
| Lenzites trabea | 539 | U.S.D.A.* |
| Merulius lacrymans | FP 94365 | " |
| Polyporus betulinus | 58514-S | " |
| Polyporus immitus | FP 71384 | " |
| Polyporus palustris | 91452 | " |
| Polyporus schweinitzii | 71356-S | " |
| Polyporus spraguei | 14857-S | " |
| Polyporus sulphureus | 48603-S | " |
| Poria cocos | 71051 | " |
| Poria incrassata | 563 | " |
| Poria luteofibrata | FP 94373-R | " |
| Poria monticola (originally microspora) | 575 | " |
| Poria nigra | 71118 | " |
| Poria oleraceae | 198 | " |
| Poria vaillantii | 90877 | " |
| Ptychogaster rubescens | UIFP 716 | " |
| Trametes malicola | 71956 | " |
| Trametes serialis | 11977 | " |
| White rots-- | | |
| Armillaria mellea | FP 46700 | " |
| Fomes annosus | 90898-R | " |
| Fomes fomentarius | 59009-S | " |
| Fomes geotropus | 55521-S | " |
| Fomes pini | 71757 | " |
| Lentinus tigrinus | 466 | " |
| Peniophora gigantea | 56475-S | " |
| Polyporus abietinus | 71429-R | " |
| Polyporus anceps | 58526 | " |
| Polyporus fumosus | # 4 | Brazil** |
| Polyporus tulipiferus | Mad. 517 | U.S.D.A.* |
| Polyporus versicolor | 57034-R | " |
| Poria subacida | 71955 | " |

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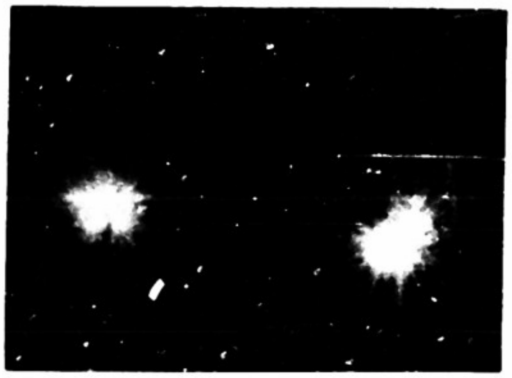
Submerged culture technique

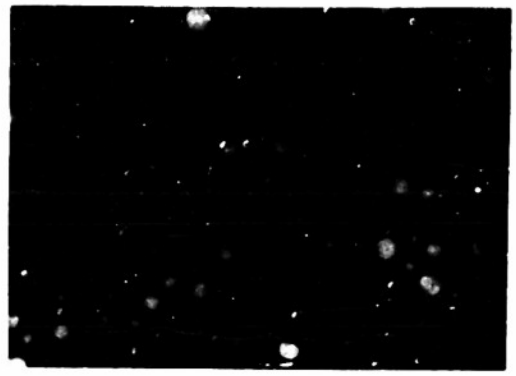
The basic routine method for growing the wood rots was that of aerated liquid culture (submerged culture), using small flasks on a shaking machine (shake culture), or large bottles with forced aeration. Reproduction of bits of the fungus results in the formation of pellets of mycelium, the form and amount of which vary with the organism and the nutrient. (Fig. 1). The submerged culture technique offers several physiologically-desirable conditions for controlled growth and, in addition, the large bottles (Fig. 2) are useful for the quantity production of mold mycelium, enzymes, or fermentation products. Most of the work was done with shake cultures (Fig. 4), using 70 ml. of culture fluid per 250-ml. Erlenmeyer flask. Solutions of the nutrients under test constituted the culture fluid. Except in studies on pH, all culture media routinely were adjusted so that they had a pH of approximately 5.5 after sterilization. The inoculated flasks were incubated on a reciprocating shaking machine (Fig. 3) having a stroke of 1½ inches and giving 110 3-inch excursions per minute. Routinely, all incubations were at 28°-30°C. for 7 days unless otherwise specified. Determinations of pH and Eh were made with a Beckman apparatus.

General procedures

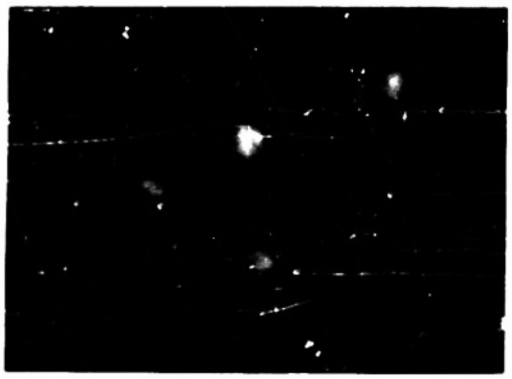
The stock cultures of the fungi were carried on potato dextrose agar (PDA), then grown in shake culture in a standard 1% malt extract (Difco, desiccated) broth to produce pellets of mycelium for inoculating the test media. A standard amount (0.15 ml., = 0.02-0.03 mg dry weight) of blended, washed growth was used for all inoculations, except the first inoculum from the PDA. The mycelial pellets were blended in a Waring blendor for 1 minute and the material in the resulting homogeneous suspension washed by centrifuging at 2000 rpm and resuspending three times. The primary



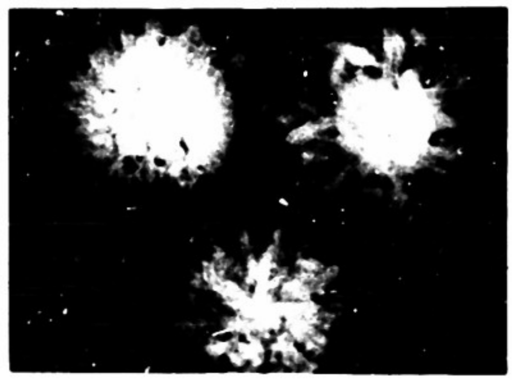
FOMES MELIAE
Malt extract



DAEDALEA QUERCINA
Malt extract



POLYPORUS PALUSTRIS
Malt extract



POLYPORUS PALUSTRIS
Bran extract

LENZITES TRABEA
Bran extract

POLYPORUS TULIPIFERUS
Bran extract

FIGURE 1 REPRESENTATIVE FORMS OF MYCELIAL PELLETS OF WOOD ROTS IN SHAKE CULTURE. TWICE NATURAL SIZE.

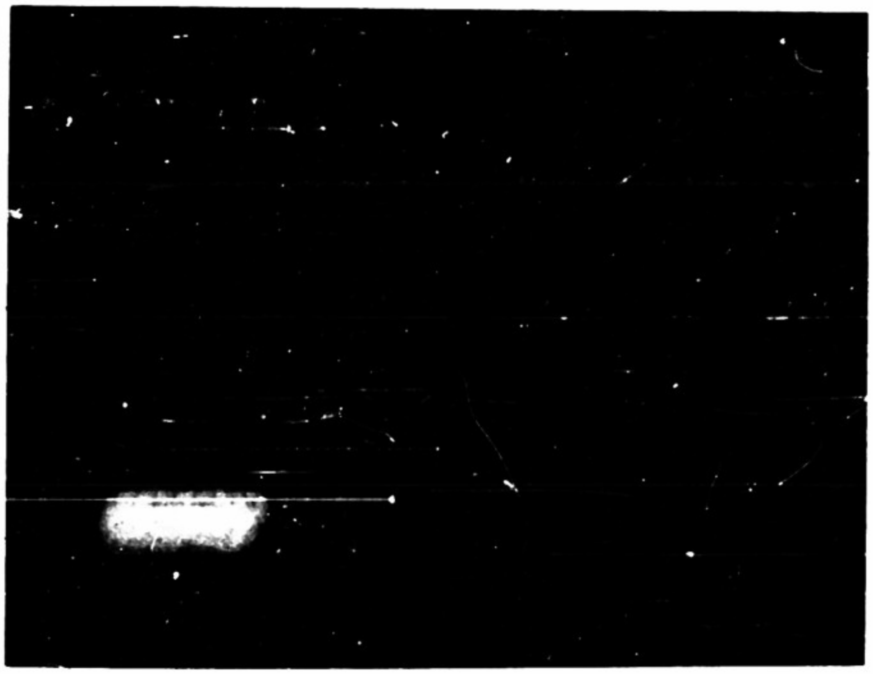


FIGURE 2. AERATED BOTTLE CULTURE OF WOOD-ROTTING FUNGUS. One liter of culture medium in 2-quart bottle. Left: glass-fiber filter for sterilizing air; right: flowmeter for measuring rate of air flow.

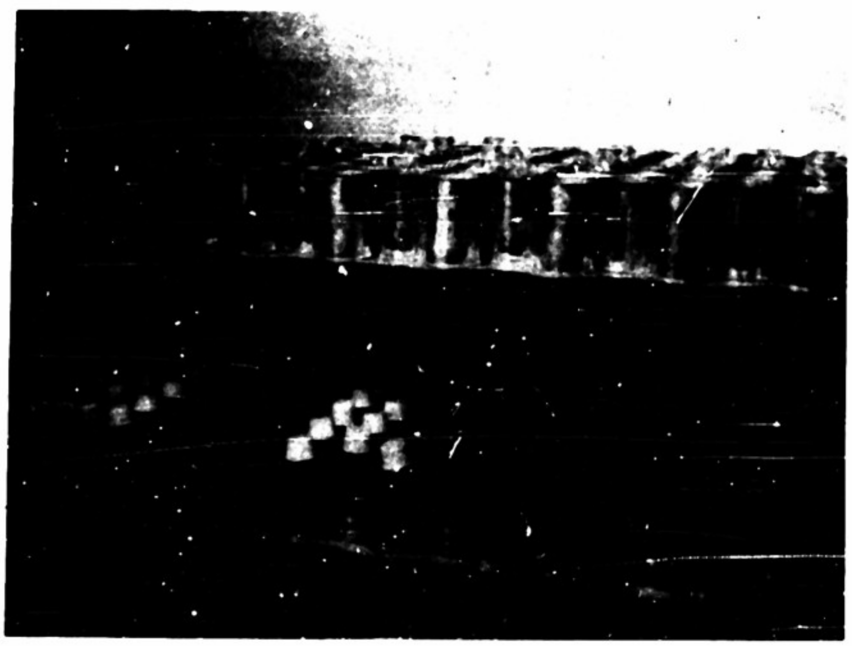


FIGURE 3. DOUBLE-DECK RECIPROCATING SHAKING MACHINE. Capacity 270 250-ml. flasks; stroke: $1\frac{1}{2}$ inches; speed: ~~120~~ 110 3-inch cycles per minute.

FIGURE 4. SHAKE CULTURES OF WOOD-ROTTING FUNGI.
70 ml. of culture medium in 250-ml. flasks. Left: a trace of growth; center: medium growth (circa 50 mg.); right: good growth (circa 100 mg.).

inoculum into a given _test_ medium was growth from the standard malt extract broth, then each of two succeeding serial subcultures _in that same test medium_ was started with washed, blended growth from the preceding one. Thus, all data on the growth of a fungus in a given nutrient are based routinely on the _third_, serial, 7-day subculture in that nutrient, using controlled inoculum size. Experiment showed that at least three such successive transfers were necessary in order to minimize or rule out the carry-over by the organism of nutrients from one medium to another. All quantitative data on the value of test nutrients are based, unless otherwise specified, on the dry weight of mycelium produced in media in duplicate flasks. (Nutritionally-adequate media resulted in the production, per 70 ml. of medium, of from 50 to 150 mg. (dry weight) of fungal mycelium from an inoculum of 0.02-0.03 mg.). Because many organisms did not attain their maximum growth in the 7-day incubation period, the growth data actually measure _rate_ of growth. The standard error of mycelial weights in duplicate flasks was about $\pm$ 4%. Flasks for the shake cultures were capped with a fresh, 4-inch square of special cellophane (Dupont, type 450 PT) which withstands autoclaving, held in place loosely by a rubber band. (This material deteriorates so that it cannot be autoclaved if more than three months old.) The use of this cellophane is much to be preferred to the usual cotton plug which may introduce traces of contaminating nutrients into the chemically defined test solutions. Also to minimize contamination with unknown nutrients, special care was used in cleaning glassware. Only distilled water was used in rinsing glassware and in making solutions, and chemicals were of the highest purity obtainable. Controls were run routinely in all tests. All media were autoclaved at 15 pounds (121°C.) for 20 minutes. For the basal medium, the inorganic salts, glucose and vitamins were sterilized separately, then combined aseptically. After autoclaving, media were cooled, shaken for 1 hour on the shaking machine, and inoculated.

Special procedures for various studies are given below in the appropriate sections.

<u>Preparation of Standard Inoculum</u>

(1) Transfer contents of one flask culture (70 ml) to a one-pint glass Waring blendor cup and grind for 1 minute

(2) Pipette 15-ml of homogenate into 15 ml graduated centrifuge tube.

(3) Centrifuge for 2 min at 2000 r.p.m., decant supernatant and pipette or pour 10 to 15 ml of sterile distilled water (use individual tubes if poured) into tube and resuspend fragments.

(4) Repeat centrifuging, decanting and resuspending procedures twice.

(5) The last resuspension is quantitative. Sterile distilled water is added to a total of 30 times the volume of packed fragments; [redacted] for example, 0.5 ml packed fragments made up to a total of 15 ml. More than 0.5 ml of packed fragments can be discarded by removing excess mycelium when decanting in the intermediate washings.

(6) Inoculum: 0.15 ml of homogenate per 70 ml of medium.

Note: The blendor cups were covered with the top half of a Petri dish (making an all-glass unit), wrapped in paper and autoclaved for 30 min at 121°C. The centrifuge tubes and tightly-fitting rubber caps were also autoclaved separately. The tubes of water were covered with cellophane or aluminum caps. Selected standard pipettes were used to insure close check of replicates.

Basal synthetic medium

Most of the nutritional studies were carried out using synthetic (chemically defined) media. The following basal medium, used routinely, supported "minimal" growth of all the organisms investigated. It was used for determining utilisation of different compounds of nitrogen, of various carbon sources (substituted for the glucose) and of vitamins, and also as the basis for the development of quantitatively "optimal" media, varying all constituents in numerous combinations.

TABLE 1

Basal Synthetic Medium

| | |
|---|---|
| Glucose | 10.0 gm./liter |
| $KH_2PO_4$ (K=430, P=342 mg./l.) | 1.5 gm./liter |
| $MgSO_4 \cdot 7H_2O$ (Mg=49.5 mg./l.) | 0.5 gm./liter |
| Thiamine monohydrochloride | 1.00 mg./liter |
| Source of nitrogen | 120 mg./liter N (see below) |
| Trace elements | |
| B (as $H_3BO_3$) | 0.10 mg./liter |
| Mn (as $MnCl_2 \cdot 4H_2O$) | 0.01 mg./liter |
| Zn (as $ZnSO_4 \cdot 7H_2O$) | 0.07 mg./liter |
| Cu (as $CuSO_4 \cdot 5H_2O$) | 0.01 mg./liter |
| Mo (as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) | 0.01 mg./liter |
| Fe (as $FeSO_4 \cdot 7H_2O$) | 0.05 mg./liter |

To this basal medium a known nitrogen compound under study was added in a concentration of 0.012% (120 mg./liter) total nitrogen, except that amino acids which were available only as racemic mixtures (DL) were used in a concentration of 0.024% total nitrogen, on the assumption that only the natural (L) form of the acid might be utilizable. Ammonium nitrate was used in a concentration to give 0.012% ammonium nitrogen. Routinely, for purposes other than the study of nitrogen compounds, glutamic acid was the usual source of nitrogen, and was added in a concentration of 1.26 gm./liter (=120 mg./liter total nitrogen).

## RESULTS

The data herein presented on the nutrition and certain aspects of the physiology of the wood rotting fungi are based chiefly on some 40,000 shake cultures.

### OPTIMUM TEMPERATURE FOR GROWTH OF WOOD ROTS

Preliminary studies and a review of the literature made when this project was started showed that 28°C was a good "average" optimum temperature for all the organisms. Because only one constant temperature room large enough for the shaking apparatus was available, this temperature has been used routinely. However, it seemed desirable to determine more closely the optimum temperature for the various species used, and data for most of these is herein presented.

The organisms were inoculated onto malt extract (1%) agar slants, and incubated at 24°, 28°, 30° and 32°C. for, usually, 7 days. The amount of growth was determined by visual observation. The apparent optimum temperature was taken as that temperature giving the greatest amount of growth, while the temperature giving the next greatest growth indicated on which side of the apparent optimum the true optimum probably lay.

Table 2 shows the experimentally-determined approximate optimum temperatures for most of the organisms. It is apparent that while the great majority have an optimum temperature close to 28°C., there are a few species for which a higher temperature is better (notably Lenzites trabea, Poria oleraceae, Poria subacida and Polyporus abietinus), and some which grow better at a lower temperature (Fomes roseus, Fomes officinalis, Poria vaillantii, Merulius lacrymans and Fomes annosus). Merulius lacrymans is the only organism which really grows poorly at 28°C. Theoretically, the optimum temperatures would not be the same in shake culture as those above-determined on slants, but a few experiments showed that this difference is not of practical experimental significance in the nutrition work.

# TABLE 2

## OPTIMUM TEMPERATURES FOR GROWTH OF WOOD ROTS

Based on visual estimation of greatest amount of mycelium produced on malt extract (1%) agar slants, adjusted to pH 5.5, and incubated for 7 days unless otherwise indicated, at 24°, 28°, 30° and 32°C. A + or – after the apparent optimum temperature figure indicates whether the next greatest amount of growth was obtained at a temperature higher (+) or lower (–) than the value given.

| Organism | Optimum Temperature °C. |
|---|---|
| **Brown rots –** | |
| Poria incrassata* | 28– |
| Poria monticola | 28– |
| Polyporus palustris | 28+ |
| Lentinus lepideus | 28+ |
| Fomes subroseus | 28+ |
| Lenzites trabea | 32 |
| Fomes roseus* | 24+ |
| Fomes meliae | 30– |
| Fomes officinalis* | 24+ |
| Poria xantha | 28+ |
| Poria cocos | 28± |
| Poria nigra | 28– |
| Poria vaillantii* | 24+ |
| Polyporus spraguei | 28+ |
| Polyporus betulinus | 28+ |
| Polyporus immitus | 28– |
| Polyporus schweinitzii | 28– |
| Lenzites saepiaria | 28– |
| Trametes serialis | 28– |
| Hydnum pulcherrimum | 28+ |
| Lenzites striata | 28+ |
| Coniophora cerebella | 28+ |
| Trametes malicola | 28+ |
| **Brown rots (cont.) –** | |
| Ptychogaster rubescens | 28+ |
| Daedalea quercina | 28+ |
| Poria oleraceae | 30+ |
| Poria luteofibrata | 28– |
| Merulius lacrymans* | 24+ |
| Polyporus sulphureus* | 28– |
| **White rots –** | |
| Polyporus tulipiferus | 28– |
| Polyporus anceps | 28– |
| Armillaria mellea* | 28± |
| Peniophora gigantea | 28+ |
| Poria subacida | 30+ |
| Fomes fomentarius | 28+ |
| Polyporus versicolor | 28± |
| Fomes annosus | 24 |
| Lentinus tigrinus | 28± |
| Fomes geotropus | 28+ |
| Polyporus abietinus | 32 |
| Fomes pini | 28+ |
| Polyporus fumosus | 28+ |

* 14-day culture.

# GROWTH OF WOOD ROTS IN NON-SYNTHETIC CULTURE MEDIA

For orientation, and as a preliminary to the development of purely synthetic (i.e., chemically-defined) media, it was desirable to determine certain growth characteristics of wood rots in non-synthetic ("natural" or artificial) media. The media used were chosen because previous work had shown that some of the wood rots made good growth in them or because of their cheapness, nutritional qualities, or other desirable characteristics.

The following nutrient solutions were used for shake cultures of the various fungi, the concentrations (on a total nitrogen basis) being shown in Table 3: malt extract (Difco, desiccated), corn steep liquor, soy bean meal + glucose, ethyl stillage (condensed molasses distillers' solubles--trade name "Curbay B-G", U.S. Industrial Chemicals), peptone (Difco), peptone + glucose, gluten (Central Scientific Co., technical grade) + glucose, and casein hydrolysate ("vitamin-free", General Biochemicals) + glucose + thiamin + inorganic salts. The "initial pH" given is that of the sterilized but _unadjusted_ medium except for the casein hydrolysate, which was adjusted. Except for casein hydrolysate alone, all nutrients contained various vitamins in unknown amounts. The vitamin added to the vitamin-free casein hydrolysate was thiamin only (1 gamma (γ) per ml. of medium), which we had previously found to be the most essential nutrilite for the growth of wood-rotting fungi. The inorganic salt mixture used in the casein hydrolysate medium is the "trace element" mixture of the basal medium (Table 1).

The following 32 representative wood rots were tested in shake culture with the above nutrients: _brown rots_--_Fomes roseus_, _Fomes subroseus_, _Fomes meliae_, _Fomes officinalis_, _Poria incrassata_, _Poria xantha_, _Poria monticola_, _Poria cocos_, _Poria nigra_, _Poria oleraceae_, _Poria vaillantii_, _Polyporus spraguei_, _Polyporus betulinus_, _Polyporus palustris_, _Polyporus immitus_, _Polyporus schweinitzii_, _Lenzites trabea_, _Lenzites saepiaria_, _Poria luteo-fibrata_, _Merulius lacrymans_, _Lentinus lepideus_, _Trametes serialis_, _Hydnum_ *pulcherrimum*, *Ptychogaster rubescens*, *Daedalea quercina*; *white rots*--*Polyporus tulipiferus*, *Polyporus versicolor*, *Peniophora gigantea*, *Armillaria mellea*, *Poria subacida*, *Fomes fomentarius*, *Fomes annosus*. Stock cultures of these organisms are carried on potato dextrose agar.

To illustrate the data obtained, results of the growth of a representative brown rot, *Poria xantha*, in different nutrients are shown in Table 3. Corresponding data have been obtained for the 31 other wood rots named above. Estimation of the amount of growth was done visually, with the greatest amount, regardless of organism or nutrient, being recorded as "4."

The results of all third-transfer observations of the growth of 32 wood rots in 8 non-synthetic media may be summarized as follows.

1. All of the fungi tested grew to a greater or lesser extent in all of the nutrient media used, but with marked differences in the amount of growth, depending upon the medium. This indicates the diversity of nutrients for minimal growth and at the same time points up the differences among various species as regards optimal nutritional requirements.

2. The fungi producing the greatest amounts of growth were *Ptychogaster rubescens* in malt extract and in gluten + glucose; *Poria vaillantii*, *Daedalea quercina* and *Polyporus betulinus* in malt extract; *Polyporus schweinitzii* in corn steep and in gluten + glucose; *Fomes officinalis* in soy bean meal + glucose; *Poria incrassata* in ethyl stillage and in gluten + glucose; *Poria xantha* and *Fomes meliae* in gluten + glucose; *Poria cocos* in ethyl stillage; and *Polyporus immitus*, *Polyporus versicolor* and *Fomes annosus* in casein hydrolysate + glucose + thiamin + inorganic salts. These results show that there are differences in optimal nutritional requirements among various species of fungi and also suggest the nutritionally "best" media of those tested.

3. A greater number of the fungi gave good growth in casein hydrolysate +

# TABLE 3

## GROWTH CHARACTERISTICS OF WOOD ROTS IN VARIOUS NON-SYNTHETIC MEDIA

Seven-day shake cultures, third transfer. Growth estimated visually; greatest amount=4. Nitrogen percentage refers to that in the solution used.

Organism: _Poria xantha_

**Malt extract 3%**
(Nitrogen = 0.012%)

| | |
|---|---|
| Amount of growth | 1 |
| Pellet size | medium |
| Initial pH | 4.4 |
| Final pH | 2.6 |

**Corn steep liquor**
(Nitrogen = 0.012%)

| | |
|---|---|
| Amount of growth | 2 |
| Pellet size | medium |
| Initial pH | 4.3 |
| Final pH | 2.6 |

**Soy bean meal + glucose 1%**
(Nitrogen = 0.012%)

| | |
|---|---|
| Amount of growth | 1 |
| Pellet size | medium |
| Initial pH | 6.0 |
| Final pH | 3.3 |

**Ethyl stillage ("Curbay B-G")**
(Nitrogen = 0.012%)

| | |
|---|---|
| Amount of growth | 2 |
| Pellet size | mixed |
| Initial pH | 5.4 |
| Final pH | 5.6 |

**Peptone 1%**
(Nitrogen = 0.16%)

| | |
|---|---|
| Amount of growth | 1 |
| Pellet size | medium |
| Initial pH | 6.6 |
| Final pH | 8.3 |

**Peptone 1% + glucose 1%**
(Nitrogen = 0.16%)

| | |
|---|---|
| Amount of growth | 1 |
| Pellet size | medium |
| Initial pH | 6.1 |
| Final pH | 4.2 |

**Gluten 1% + glucose 1%**
(Nitrogen = 0.12%)

| | |
|---|---|
| Amount of growth | 3 |
| Pellet size | small |
| Initial pH | 6.7 |
| Final pH | 1.8 |

**Casein hydrolysate + glucose 1% + salts + thiamin (1γ/ml.)**
(Nitrogen = 0.012%)

| | |
|---|---|
| Amount of growth | 2 |
| Pellet size | small |
| Initial pH | 5.2 |
| Final pH | 4.0 |

glucose + thiamin + inorganic salts than in any other medium; gluten + glucose, and ethyl stillage were close seconds. Peptone, and peptone + glucose were the poorest media as regards the majority of fungi, while malt extract, corn steep, and soy bean meal + glucose were intermediate for the greatest number of fungi. These data (with controls) show that thiamin is necessary for most of the fungi used, and suggest that it is the only essential vitamin. It also is clear that certain of the "natural" or artificial nutrient mixtures are not in general as good "balanced" diets as others.

4. In general, as a result of the metabolism of the fungi in shake culture, there is an increase in acidity of the medium. Often this is quite marked; a pH of around 2 is frequently attained, particularly in the media containing added sugar. The lowest pH values reached were not uniformly characteristic of any particular species of organisms or kind of nutrient.

5. The chief exception to the generalization that acid is produced in shake culture is, as one might expect, the peptone medium. This is the only nutrient medium which does not contain sugar. The final pH in this medium is alkaline in the case of about half of the species of fungi used, and only slightly acid with the remainder. Presumably this results from the production of ammonia.

6. The size of the pellets of growth appears, in general, to be more closely correlated with the type of nutrient than with the species of organism. For example, pellets of growth in malt extract were invariably small for all species of fungi. In corn steep, a good majority of the 32 species formed small pellets, although a few produced pellets of medium size. In the 6 other nutrients, the pellet size was small for about half of the fungi, and larger with the remainder. There was little tendency, however, for a given species to produce the same size of pellet in different media.

7. Although the form in which the nitrogen occurs is not known for all of the media used, it is clear from the results with casein hydrolysate that the fungi can utilize nitrogen in a form at least as simple as amino acids and/or polypeptide

PRELIMINARY EXPERIMENTS ON GROWTH IN SYNTHETIC CULTURE MEDIA

This section deals with orientation experiments on the development of synthetic, i.e., chemically-defined, media for growth of the wood rots. Definition of the nutritional requirements of these fungi in terms of known chemical compounds, and particularly their nitrogen and vitamin requirements, is basic to an understanding of their activities in attacking wood, and to a study of other aspects of their physiology. The general approach in this work has been that of trying progressively simpler nitrogen compounds, together with various nutrilites. All of this work was done using shake cultures as described in the section above and, for the reason previously explained, all data are third-subculture observations.

In the development of the synthetic media, only two representative wood rots were studied in detail: Polyporus palustris, a brown rot, and Polyporus tuliniferus, a white rot. The amount of growth produced after 7 days, with our standard shake culture technique, was used as a measure of the nutritive value of the synthetic media. Since the maximum amount of growth is not necessarily attained within the 7-day period, we are actually basing our comparisons on rates of growth. Measurement of the amount of 7-day-old growth in the various media has been quantitative throughout, using, in each case, the oven-dry weight of mycelial pellets produced in our standard shake flask with 70 ml. of medium. This weight is expressed as the percentage of the oven-dry mycelial weight formed by the same organism in 1% malt extract medium, which is a non-synthetic nutrient. (The oven-dry weight of mycelial pellets obtained after 7 days in our standard shake culture averaged 0.1027 gm. for P. palustris, and 0.0694 gm. for P. tuliniferus, with a standard error of ±3-4%). The amount of growth obtained in 1% malt extract (total nitrogen = 0.004%)

is used merely as a datum point for comparing the synthetic media among themselves on an equivalent total nitrogen basis (0.012%). Growth in 3% malt extract (total nitrogen = 0.012%) is too gummy to be separated from the medium for accurate weighing.

For comparing synthetic media, a basal medium of glucose (sterilized separately) and inorganic salts was used in every case (Table 1). To this basal medium the other constituents (nitrogen sources and nutrilites) were added, thiamin being sterilized separately. The inorganic salt mixture was arrived at empirically, and while satisfactory for the time being, is not necessarily optimal in all respects.

Because of the relatively good growth of all of the wood rots in the casein hydrolysate medium (see preceding section), this nitrogen source was used as the starting point in developing purely synthetic nutrient mixtures. (Actually, casein hydrolysate is a semi-synthetic medium, because the mixture of amino acids is not clearly defined, but it serves as a good basis for further work.) Starting with casein hydrolysate, a number of other organic and inorganic nitrogen sources have been tested, with various nutrilites--vitamins, and purine and pyrimidine bases--in numerous combinations.

The complete list of nitrogen sources so far investigated is as follows: casein hydrolysate ("vitamin-free" General Biochemicals), urea, sodium glutamate, l(+)-arginine (monohydrochloride), l-asparagine, l(+)-cysteine (hydrochloride), l(+)-histidine (monohydrochloride), dl-methionine, dl-ornithine (hydrochloride), dl-serine, dl-valine. These nitrogen sources were always added to the basal medium so as to give a total nitrogen of 0.012%. The nutrilites (vitamins, purine and pyrimidine bases) used in various combinations were: vitamins--thiamin (hydrochloride), biotin, pyridoxine (hydrochloride), i-inositol, calcium panthothenate, riboflavin, niacin (nicotinic acid), folic acid (pteroylglutamic acid), choline(chloride); _purine bases_--guanine (hydrochloride), adenine (sulfate), xanthine; _pyrimidine bases_--uracil. These nutrilites were used, for the time being, in the following more or less arbitrary, but excess, concentrations: thiamin, riboflavin, niacin, calcium pantothenate and pyridoxine -- 1 gamma per _ml_. of medium; choline and inositol -- 2 gammas per _ml_. of medium; guanine, adenine and uracil -- 13 mg per _liter_ of medium; xanthine -- 20 mg. per _liter_ of medium; biotin and folic acid -- 2 gammas per _liter_ of medium.

All media were adjusted so as to give, after sterilisation, a pH of about 5.5, which we have found to be the approximately optimum pH for the growth of the organisms used. No attempt was made to buffer the medium. Reference in the tables to "any" or to "all" vitamins or other nutrilites means, of course, those listed above.

Table 4 is a very condensed summary of typical average results of third-subculture observations on P. palustris in synthetic media containing the several nitrogen sources tested with various combinations of nutrilites. The standard error of duplicates varied between 0.4 and 6.4%, averaging ± 3.2%. In Table 5 similar data are given for P. tulipiferus. Because the over-all data are as yet incomplete, the summary must necessarily apply only to the results so far obtained.

The main points to date regarding the nutrition of P. palustris and P. tulipiferus may be briefly summarized from Tables 4 and 5 as follows:

1. Neither of these fungi will grow in the complete absence of added nutrilites in any of the nitrogen sources tried.

2. Continued growth in subculture of either organism is obtained in any of the nitrogen sources except sodium nitrate, in the presence of thiamin or biotin.

3. Apparently thiamin and biotin are qualitatively interchangeable in the nutrition of either of these fungi.

4. Growth is considerably greater with thiamin alone than with biotin alone, when the vitamin is present in excess.

5. Growth in any or all nutrilites other than thiamin or biotin is very meager and is not long-continued in subculture.

6. The use of certain other nutrilites added to thiamin or biotin often stimulates growth, which in some cases is strikingly in excess of that obtained using the same other nutrilites alone, or thiamin or biotin alone. The effect of purine or pyrimidine bases plus thiamin is especially noteworthy in this regard

7. For maximal growth, other nutrilites are needed with thiamin or biotin; continued growth -- although smaller in amount -- is obtained with either of the latter alone.

8. No nutrilite other than thiamin or biotin appears to be essential for continued growth, and no other can replace thiamin or biotin.

TABLE 4

GROWTH OF P. PALUSTRIS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thiamin only | | | Biotin only | | | Thiamin plus any other vitamin except inositol or folic acid | | | Thiamin plus inositol or folic acid | | |
| Nitrogen Source | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH |
| Casein hydrolysate | 57 | 5.4 | 1.8 | 45 | 5.3 | 1.8 | 62 | 5.4 | 1.5 | 38 | 5.4 | 1.9 |
| Urea | 25 | 5.4 | 1.8 | 20 | 5.5 | 2.2 | 26 | 5.5 | 1.9 | 26 | 5.5 | 1.8 |
| Na glutamate | 75 | 5.4 | 1.7 | 38 | 5.4 | 2.1 | 40 | 5.3 | 1.9 | | | |
| $(NH_4)_2CO_3$ | 50 | 5.5 | 1.9 | 61 | 5.5 | 1.6 | 60 | 5.5 | 1.8 | | | |
| Na $NO_3$ | 0 | 5.5 | 5.4 | 0 | 5.5 | 5.4 | 0 | 5.5 | 5.4 | | | |
| Arginine | 60 | 5.3 | 1.4 | | | | | | | | | |
| Asparagine | 79 | 5.3 | 1.8 | | | | | | | | | |
| Cysteine | 20 | 5.2 | 2.5 | | | | | | | | | |
| Histidine | 38 | 5.2 | 1.7 | | | | | | | | | |
| Methionine | 20 | 5.3 | 2.3 | | | | | | | | | |
| Ornithine | 58 | 5.3 | 1.9 | | | | | | | | | |
| Serine | 60 | 5.3 | 1.9 | | | | | | | | | |
| Valine | 60 | 5.3 | 1.7 | | | | | | | | | |

TABLE 4

GROWTH OF P. PALUSTRIS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thiamin and biotin plus <u>any</u> other vitamin <u>except</u> inositol, folic acid, or choline | | | Thiamin plus <u>all</u> other vitamins <u>except</u> inositol, folic acid, or choline | | | <u>Any</u> or all vitamins <u>except</u> thiamin and biotin | | | Thiamin plus any purine or pyrimidine base | | |
| <u>Nitrogen Source</u> | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Inital pH | Final pH | Growth % | Initial pH | Final pH |
| Casein hydrolysate | 66 | 5.5 | 1.6 | 70 | 5.4 | 1.6 | 6 | 5.5 | 5.0 | 89 | 5.4 | 1.7 |
| Urea | 26 | 5.5 | 1.8 | 30 | 5.4 | 1.8 | 12 | 5.5 | 5.2 | 40 | 5.3 | 1.9 |
| Na glutamate | | | | | | | | | | | | |
| $(NH_4)_2CO_3$ | | | | 61 | 5.5 | 1.8 | 0 | 5.5 | 5.5 | 83 | 5.3 | 1.7 |
| Na $NO_3$ | | | | 0 | 5.4 | 5.4 | 0 | 5.5 | 5.5 | | | |
| Arginine | | | | | | | | | | | | |
| Asparagine | | | | | | | | | | | | |
| Cysteine | | | | | | | | | | | | |
| Histidine | | | | | | | | | | | | |
| Methionine | | | | | | | | | | | | |
| Ornithine | | | | | | | | | | | | |
| Serine | | | | | | | | | | | | |
| Valine | | | | | | | | | | | | |

TABLE 4 (CONT.)

GROWTH OF P. PALUSTRIS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | |
|---|---|---|---|---|---|---|---|
| | Thiamin plus all other vitamins and all bases | | | Any or all bases only | | | No nutrilites |
| Nitrogen Source | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % |
| Casein hydrolysate | 92 | 5.5 | 1.6 | 8 | 5.4 | 2.6 | 0 |
| Urea | 43 | 5.5 | 1.7 | 0 | 5.4 | 5.4 | 0 |
| Na glutamate | | | | | | | |
| $(NH_4)_2CO_3$ | 96 | 5.4 | 1.4 | | | | 0 |
| $NaNO_3$ | 0 | 5.4 | 5.4 | | | | 0 |
| Arginine | | | | | | | |
| Asparagine | | | | | | | |
| Cysteine | | | | | | | |
| Histidine | | | | | | | |
| Methionine | | | | | | | |
| Ornithine | | | | | | | |
| Serine | | | | | | | |
| Valine | | | | | | | |

TABLE 5

GROWTH OF P. TULIPIFERUS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thiamin only | | | Biotin only | | | Thiamin plus <u>any</u> other vitamin <u>except</u> inositol | | | Thiamin plus inositol | | |
| | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH |
| <u>Nitrogen Source</u> | | | | | | | | | | | | |
| Casein hydrolysate | 48 | 5.4 | 2.7 | 25 | 5.5 | 3.8 | 51 | 5.4 | 4.5 | 36 | 5.4 | 3.2 |
| Urea | 13 | 5.4 | 4.1 | 6 | 5.5 | 4.0 | 14 | 5.5 | 4.3 | 14 | 5.4 | 4.3 |
| Na glutamate | 60 | 5.4 | 4.8 | 20 | 5.4 | 4.1 | 60 | 5.3 | 4.2 | | | |
| $(NH_4)_2CO_3$ | 40 | 5.5 | 4.4 | 30 | 5.3 | 4.5 | 42 | 5.5 | 4.6 | | | |
| $Na\ NO_3$ | 0 | 5.5 | 5.5 | 0 | 5.5 | 5.5 | 0 | 5.5 | 5.4 | | | |
| Arginine | 40 | 5.3 | 4.8 | | | | | | | | | |
| Asparagine | 40 | 5.2 | 4.9 | | | | | | | | | |
| Cysteine | 20 | 5.2 | 3.4 | | | | | | | | | |
| Histidine | 30 | 5.3 | 3.9 | | | | | | | | | |
| Methionine | 30 | 5.3 | 4.0 | | | | | | | | | |
| Ornithine | 40 | 5.3 | 5.0 | | | | | | | | | |
| Serine | 40 | 5.3 | 4.5 | | | | | | | | | |
| Valine | 60 | 5.3 | 4.1 | | | | | | | | | |

TABLE 5 (CONT.)

GROWTH OF P. TULIPIFERUS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Thiamin <u>and</u> biotin plus <u>any</u> other vitamin <u>except</u> inositol, folic acid, or choline | | | Thiamin plus <u>all</u> other vitamins <u>except</u> inositol, folic acid, or choline | | | <u>Any</u> or <u>all</u> vitamins <u>except</u> thiamin and biotin | | | Thiamin plus any purine or pyrimidine base | | |
| | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH |
| <u>Nitrogen Source</u> | | | | | | | | | | | | |
| Casein hydrolysate | 54 | 5.5 | 4.3 | 62 | 5.4 | 3.8 | 10 | 5.4 | 3.6 | 81 | 5.4 | 4.4 |
| Urea | 16 | 5.5 | 4.1 | 22 | 5.5 | 3.9 | 6 | 5.5 | 3.9 | 38 | 5.3 | 4.2 |
| Na glutamate | | | | | | | | | | | | |
| $(NH_4)_2CO_3$ | | | | 44 | 5.5 | 4.2 | 0 | 5.5 | 5.5 | 68 | 5.5 | 4.8 |
| Na $NO_3$ | | | | 0 | 5.4 | 5.4 | 0 | 5.5 | 5.5 | | | |
| Arginine | | | | | | | | | | | | |
| Asparagine | | | | | | | | | | | | |
| Cysteine | | | | | | | | | | | | |
| Histidine | | | | | | | | | | | | |
| Methionine | | | | | | | | | | | | |
| Ornithine | | | | | | | | | | | | |
| Serine | | | | | | | | | | | | |
| Valine | | | | | | | | | | | | |

TABLE 5 (CONT).

GROWTH OF P. TULIPIFERUS IN SYNTHETIC MEDIA

Basal medium plus nitrogen sources and nutrilites as indicated. Seven-day shake cultures, third transfer. Growth (dry weight) expressed as percentage of that obtained in 1% malt extract.

| | Nutrilite | | | | | | |
|---|---|---|---|---|---|---|---|
| | Thiamin plus all other vitamins and all bases except inositol, folic acid or choline | | | Any or all bases only | | | No nutrilites |
| Nitrogen Source | Growth % | Initial pH | Final pH | Growth % | Initial pH | Final pH | Growth % |
| Casein hydrolysate | 91 | 5.5 | 5.4 | 8 | 5.4 | 5.0 | 0 |
| Urea | 42 | 5.5 | 3.9 | 0 | 5.5 | 5.5 | 0 |
| Na glutamate | | | | | | | |
| $(NH_4)_2CO_3$ | 73 | 5.4 | 4.5 | | | | 0 |
| $NaNO_3$ | 0 | 5.4 | 5.4 | | | | 0 |
| Arginine | | | | | | | |
| Asparagine | | | | | | | |
| Cysteine | | | | | | | |
| Histidine | | | | | | | |
| Methionine | | | | | | | |
| Ornithine | | | | | | | |
| Serine | | | | | | | |
| Valine | | | | | | | |

9. The combination of folic acid and/or inositol with thiamin appears to have some inhibitory effect on growth.

10. The amount of growth obtained from P. palustris is fairly consistently greater than that from P. tulipiferus, under the same conditions.

11. P. palustris consistently produces a lower pH than does P. tulipiferus.

12. In general, and except for urea, organic nitrogen sources result in better growth than inorganic nitrogen compounds.

13. The data so far indicate that these organisms cannot reduce nitrate nitrogen to amino nitrogen, but can use ammonia nitrogen for protein synthesis.

14. The organisms produced about one-half as much growth in the urea as in the ammonium carbonate. This suggests that possibly only one of the amino nitrogens of the urea can be utilized by the organisms; or it may be due to the known toxic effect of urea; or it may be due to the higher carbon dioxide of the ammonium carbonate.

15. The greatest amount of growth (and lowest pH) was obtained with P. palustris in ammonium carbonate plus all vitamins and nitrogen bases. (Table 4). This suggests that a high carbon dioxide tension or a higher available carbon content is helpful, as the carbonate was probably mostly carbon dioxide at the p H of 1.4 which was attained.

16. Of the single amino acids used as nitrogen sources, the least growth is obtained with cysteine or methionine. It may or may not be significant that these are the only sulfur-containing amino acids of these tried.

The effect of different concentrations of nutrilites in synthetic media was explored somewhat before selecting the arbitrary but excess amounts actually used. The concentrations of nutrilites used in Tables 4 and 5 represent roughly the "optimum" levels for maximum growth of *P. palustris* and *P. tulipiferus* in casein hydrolysate medium. They were determined by using a range of concentration and selecting the concentration giving the greatest amount of growth. In some cases even a two-fold change in nutrilite concentration resulted in a marked difference in growth; in other cases the difference was not measurable. For example, increasing the concentration of biotin from 1 gamma to 4 gammas per liter slightly increased the growth of *P. palustris*, but more than cut in half the growth of *P. tulipiferus*. With thiamin, a decrease from 1 gamma to 0.5 gamma per ml. had no effect on *P. palustris*, but cut in half the growth of *P. tulipiferus*. Further quantitative study of nutrilites will be found in a later section.

## EFFECT OF CONTINUED SERIAL SUBCULTURE

Upon the first subculture of an organism from one medium to another of different composition, some growth often occurs even if the new medium lacks essential nutrients. This has been called "growth momentum," and is due to the carry-over by the organism (inoculum) of small amounts of essential nutritive substances. Thus growth in the first subculture should never be taken as an indication of the nutritional adequacy of the new medium. Similarly, one sometimes obtains growth in the second subculture, i.e., the first transfer from the new medium into another portion of the same medium. Less often does the carry-over of nutrients extend into the third subculture, hence it is generally considered safe to base observations of growth in the new medium on the third subculture.

To determine the necessity for successive serial subculture before drawing conclusions regarding growth in a new medium, typical quantitative observations for 8 such transfers are illustrated in Table 6. In each case the original inoculum was that grown in 1% malt extract medium. "Subculture #1" refers to the growth, obtained in the designated synthetic media, which resulted from the original inoculum. "Subculture #2" is the growth, in the designated media, which resulted from using as an inoculum a portion of the growth in "subculture #1"; and so on.

The data in Table 6 demonstrate the necessity for using at least third-subculture observations as the basis for drawing quantitative conclusions regarding growth even in a nutritionally-adequate medium. The amount of growth is greatest in the first subculture (because of "growth momentum"); it decreases from the first to about the third transfer, then levels off and continues approximately constant "indefinitely." (In a nutritionally-inadequate medium, growth would decrease, then stop).

TABLE 6

EFFECT OF CONTINUED SUBCULTURE IN SYNTHETIC MEDIA

Growth (dry weight) expressed as percentage of that obtained in 1% malt extract. Original inoculum was growth from malt extract.

| Nutrilite | Subculture | Casein hydrolysate as nitrogen source: P. palustris—% growth | Casein hydrolysate as nitrogen source: P. tulipiferus—% growth | Urea as nitrogen source: P. palustris—% growth | Urea as nitrogen source: P. tulipiferus—% growth |
|---|---|---|---|---|---|
| Thiamin | 1 | 65 | 70 | 32 | 20 |
| | 2 | 60 | 55 | 28 | 13 |
| | 3 | 57 | 48 | 25 | 13 |
| | 4 | 55 | 44 | 24 | 13 |
| | 5 | 55 | 47 | 20 | 13 |
| | 6 | 54 | 47 | 22 | 12 |
| | 7 | 55 | 47 | 22 | 10 |
| | 8 | 57 | 47 | 22 | 10 |
| Biotin | 1 | 48 | 35 | 22 | |
| | 2 | 47 | 28 | 20 | |
| | 3 | 45 | 25 | 20 | |
| | 4 | 40 | 26 | 20 | |
| | 5 | 35 | 24 | 18 | |
| | 6 | 32 | 21 | -- | |
| | 7 | 32 | 16 | 20 | |
| | 8 | 32 | 15 | 20 | |
| Thiamin, biotin, niacin, riboflavin, calcium panto-thenate, pyridoxine | 1 | 82 | 75 | 35 | 28 |
| | 2 | 69 | 65 | 32 | 23 |
| | 3 | 70 | 62 | 30 | 22 |
| | 4 | 72 | 62 | 28 | 20 |
| | 5 | 74 | 62 | 25 | 23 |
| | 6 | 70 | 60 | 25 | 21 |
| | 7 | 70 | 60 | 25 | 22 |

## OPTIMUM pH FOR GROWTH OF WOOD ROTS

The approximately optimum pH for the growth of 42 species of wood rots was determined in shake culture in the basal medium plus glutamic acid, as indicated by the amount of mycelium produced in, usually, 7 days. The initial pH was varied from 3.5 to 6.0, in steps of 0.5 pH, for each organism. The results are shown in Table 7.

It can be seen from this table that while all organisms give good growth at the "standard" pH of 5.5, which has been used for most of the work on nutrition, a few species have an optimum as low or lower than 3.5. This means that the quantitative data in Tables 10 and 11, below, are strictly comparable only at the pH (5.0 - 5.5) used, not at the optimum pH.

TABLE 7

OPTIMUM pH FOR GROWTH OF WOOD ROTS IN SHAKE CULTURE AT 28°C., AND GROWTH AT OPTIMUM pH COMPARED WITH THAT AT pH 5.5 (STANDARD)

Based on dry weight of mycelium produced in 70 ml. of basal medium (Table 1) plus glutamic acid (to 0.012% nitrogen), with initial pH adjusted to approximately 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0, respectively. Standard inoculum used. Data are for at least duplicate, 7-day, third-transfer cultures unless otherwise indicated. Growth at optimum pH expressed as percentage of that at standard pH 5.5 (medium not adjusted). A + or - after the optimum pH figures indicates whether the next greatest amount of growth was obtained at a more alkaline (+) or a more acid (-) pH than the value given.

| Organism | Optimum pH (± 0.5 pH) | A Mat weight, optimum pH mg. | B Mat weight, pH 5.5 mg. | $\frac{A}{B}$ x 100 % |
|---|---|---|---|---|
| Brown rots - | | | | |
| Poria incrassata | 6.0 * | 52.1° | 44.6° | 117 |
| Poria monticola | 5.5- | 87.6 | 87.6 | 100 |
| Polyporus palustris | 5.5- | 91.3 | 91.3 | 100 |
| Lentinus lepideus | 3.5 * | 67.8° | 10.5° | 646 |
| Fomes subroseus | 4.5- | 80.1 | 72.0 | 111 |
| Lenzites trabea | 4.0+ | 90.6 | 85.0 | 107 |
| Fomes roseus | 3.5 * | 61.2° | 42.6° | 144 |
| Fomes meliae | 5.5- | 96.0 | 96.0 | 100 |
| Fomes officinalis ** | 4.5+ | 67.8° | 62.5° | 109 |
| Poria xantha | 5.5- | 54.7 | 54.7 | 100 |
| Poria cocos | 4.5+ | 20.1 | 10.2 | 196 |
| Poria nigra | 5.0+ | 152.9 | 140.2 | 109 |
| Poria vaillantii ** | 5.0- | 99.6° | 92.4° | 108 |
| Polyporus spraguei | 5.0- | 39.2 | 27.3 | 144 |
| Polyporus betulinus | 4.5+ | 87.6 | 82.0 | 107 |
| Polyporus immitus | 5.0- | 42.8°° | 36.2°° | 118 |
| Trametes malicola | 3.5 * | 68.7 | 42.3 | 162 |

* End of pH-series used, hence direction of true optimum pH not known.
** Grown in 1% malt extract.
° 14-day culture.
°° 24-day culture.

(continued on next page)

TABLE 7 (CONCLUDED)

| Organism | Optimum pH (± 0.5 pH) | A Mat weight optimum pH mg. | B Mat weight pH 5.5 mg. | $\frac{A}{B}$ x 100 % |
|---|---|---|---|---|
| Brown rots (cont.) - | | | | |
| Polyporus schweinitzii | 5.0+ | 56.4° | 40.1° | 141 |
| Lenzites saepiaria | 5.0- | 22.4 | 20.1 | 112 |
| Trametes serialis | 4.5- | 72.4 | 65.3 | 111 |
| Hydnum pulcherrimum | 5.0+ | 32.1° | 29.8° | 108 |
| Ptychogaster rubescens | 4.0- | 107.3 | 63.9 | 168 |
| Daedalea quercina | 4.0- | 40.2 | 20.2 | 200 |
| Poria oleraceae | 5.0- | 90.5 | 42.6 | 212 |
| Poria luteofibrata | 5.0+ | 30.2°° | 28.2°° | 107 |
| Merulius lacrymans | 4.5- | 24.2 | 18.7 | 130 |
| Polyporus sulphureus | 5.5- | 49.6°° | 49.6°° | 100 |
| Lenzites striata | 5.0+ | 33.8 | 31.6 | 107 |
| Coniophora cerebella | 4.0- | 42.7 | 24.6 | 174 |
| White rots - | | | | |
| Polyporus tulipiferus | 5.5- | 87.2 | 87.2 | 100 |
| Polyporus anceps | 5.0+ | 53.8 | 52.7 | 102 |
| Armillaria mellea | 4.5+ | 26.4° | 16.4° | 161 |
| Peniophora gigantea | 5.5- | 98.2 | 98.2 | 100 |
| Poria subacida | 3.5 * | 82.6° | 58.2° | 142 |
| Fomes fomentarius | 5.0+ | 5.9 | 2.6 | 226 |
| Polyporus versicolor | 5.5± | 98.2 | 98.2 | 100 |
| Fomes annosus | 5.5+ | 63.2° | 63.2° | 100 |
| Lentinus tigrinus | 5.5+ | 98.3 | 98.3 | 100 |
| Fomes geotropus | 5.0- | 55.3 | 50.2 | 110 |
| Polyporus abietinus | 5.5- | 29.2 | 29.2 | 100 |
| Fomes pini | 5.0+ | 34.7 | 32.4 | 107 |
| Polyporus fumosus | 4.5+ | 21.3 | 15.3 | 139 |

* End of pH-series used, hence direction of true optimum pH not known.
° 14-day culture.
°° 24-day culture.

GROWTH CURVES OF THE WOOD ROTS

The increase in cell substance with time, expressed as dry weight of mycelial pellets per 70 ml. of culture medium, has been determined for 42 of the fungi in two media -- malt extract (1%), and the synthetic medium (Table 1) with L-glutamic acid (nitrogen level 0.012%) plus thiamine. All work was done in standard shake culture at 28°C., and growth was allowed to continue until a maximum was reached. Determinations of growth and of pH were made every two days, the whole contents of duplicate flasks being used at each sampling period. The results give a picture of the relative rates and amounts of growth and acid production for different organisms in the two media. Table 8 summarizes a portion of the data for 1% malt extract, and compares the mycelial weights in 7 days (our standard growth time for nutritional studies) with the maximum growth attained and the time required to reach this maximum. Also, the maximum and the terminal (at point of maximum growth) acidities attained, expressed as pH, are shown for their respective time periods. Table 9 summarizes similar data for L-glutamic acid as the nitrogen source.

TABLE 8

COMPARISON OF SEVEN-DAY GROWTH AND MAXIMUM GROWTH IN MALT EXTRACT

Growth given as milligrams of dry weight of mycelium per 70 ml. of medium, averages of duplicate flasks. "Terminal" acidity means that at the time of maximum mycelial weight.

| Organism | Seven-day mycelial weight | Maximum mycelial weight | | Maximum acidity | | Terminal acidity | |
|---|---|---|---|---|---|---|---|
| Brown rots - | mg. | mg. | days | pH | days | pH | days |
| Poria incrassata | 80 | 93 | 12 | 3.00 | 6 | 3.29 | 12 |
| Poria monticola | 116 | 131 | 12 | 2.06 | 6 | 3.03 | 12 |
| Polyporus palustris | 110 | 114 | 8 | 1.65 | 12 | 1.75 | 8 |
| Lentinus lepideus | 66 | 80 | 18 | 1.55 | 20 | 1.59 | 18 |
| Fomes subroseus | 99 | 104 | 10 | 4.05 | 10 | 4.06 | 10 |
| Lenzites trabea | 54 | 71 | 14 | 3.45 | 20 | 3.75 | 14 |
| Fomes roseus | 106 | 116 | 10 | 2.28 | 14 | 2.30 | 10 |
| Fomes meliae | 126 | 125 | 8 | 2.15 | 20 | 2.32 | 8 |
| Fomes officinalis | 63 | 71 | 14 | 2.26 | 14 | 2.26 | 14 |
| Poria xantha | 72 | 90 | 12 | 3.50 | 10 | 3.51 | 12 |
| Poria cocos | 48 | 74 | 16 | 2.83 | 18 | 2.86 | 16 |
| Poria nigra | 147 | 155 | 10 | 2.27 | 16 | 3.11 | 10 |
| Poria vaillantii | 96 | 120 | 14 | 1.75 | 18 | 1.82 | 14 |
| Polyporus immitus | 88 | 95 | 10 | 2.50 | 8 | 2.42 | 10 |
| Poria oleraceae | 123 | 138 | 10 | 2.17 | 10 | 2.17 | 10 |
| Poria luteofibrata | 12 | 43 | 40+ | 1.50 | 20 | 1.50 | 40 |
| Merulius lacrymans | 30 | 59 | 40+ | 2.82 | 8 | 3.50 | 40 |
| Polyporus sulphureus | 30 | 52 | 18 | 3.00 | 20 | 3.26 | 18 |
| Lenzites striata | 65 | 86 | 12 | 3.22 | 20 | 3.90 | 12 |
| Coniophora cerebella | 79 | 132 | 16 | 2.99 | 12 | 3.29 | 16 |
| Trametes malicola | 65 | 70 | 8 | 1.65 | 18 | 1.90 | 8 |

+ maximum growth not yet reached.

TABLE 8 (CONTINUED)

COMPARISON OF SEVEN-DAY GROWTH AND MAXIMUM GROWTH IN MALT EXTRACT

Growth given as milligrams of dry weight of mycelium per 70 ml. of medium, averges of duplicate flasks. "Terminal" acidity means that at the time of maximum mycelial weight.

| Organism | Seven-day mycelial weight | Maximum mycelial weight | | Maximum acidity | | Terminal acidity | |
|---|---|---|---|---|---|---|---|
| Brown rots (con't)- | mg. | mg. | days | pH | days | pH | days |
| Polyporus spraguei | 115 | 132 | 10 | 3.03 | 12 | 3.05 | 10 |
| Polyporus betulinus | 80 | 91 | 12 | 2.40 | 16 | 2.49 | 12 |
| Polyporus schweinitzii | 57 | 75 | 16 | 4.15 | 20 | 4.17 | 16 |
| Lenzites saepiaria | 83 | 94 | 10 | 4.30 | 12 | 4.37 | 10 |
| Trametes serialis | 221 | 240 | 10 | 4.51 | 10 | 4.51 | 10 |
| Hydnum pulcherrimum | 82 | 92 | 14 | 2.38 | 16 | 2.46 | 14 |
| Ptychogaster rubescens | 89 | 102 | 14 | 3.70 | 16 | 3.95 | 14 |
| Daedalea quercina | 113 | 126 | 12 | 2.01 | 14 | 2.21 | 12 |
| White rots - | | | | | | | |
| Polyporus tulipiferus | 130 | 137 | 10 | 3.90 | 4 | 4.45 | 10 |
| Polyporus anceps | 102 | 114 | 8 | 2.60 | 8 | 2.60 | 8 |
| Peniophora gigantea | 97 | 117 | 14 | 2.76 | 18 | 2.91 | 14 |
| Poria subacida | 97 | 115 | 12 | 3.54 | 12 | 3.54 | 12 |
| Fomes annosus | 94 | 122 | 20+ | 3.50 | 20 | 3.50 | 20 |
| Lentinus tigrinus | 100 | 121 | 14 | 4.32 | 14 | 4.32 | 14 |
| Fomes fomentarius | 71 | 85 | 10 | 3.47 | 8 | 4.06 | 10 |
| Polyporus versicolor | 118 | 151 | 10 | 3.74 | 6 | 4.14 | 10 |
| Fomes geotropus | 123 | 161 | 12 | 4.32 | 8 | 4.50 | 12 |
| Polyporus abietinus | 153 | 277 | 20+ | 3.89 | 6 | 4.61 | 20 |
| Fomes pini | 60 | 92 | 14 | 3.95 | 12 | 3.99 | 14 |
| Polyporus fumosus | 64 | 84 | 12 | 1.50 | 10 | 1.50 | 12 |
| Armillaria mellea | 11 | 22 | 20+ | 4.82 | 16 | 4.90 | 20 |

+ maximum growth not yet reached.

TABLE 9

COMPARISON OF SEVEN-DAY GROWTH AND MAXIMUM GROWTH IN GLUTAMIC ACID

Growth given as milligrams of dry weight of mycelium per 70 ml. of medium, averages of duplicate flasks. "Terminal" acidity means that at the time of maximum mycelial weight.

| Organism | Seven-day mycelial weight | Maximum mycelial weight | | Maximum acidity | | Terminal acidity | |
|---|---|---|---|---|---|---|---|
| Brown rots - | mg. | mg. | days | pH | days | pH | days |
| Poria incrassata | 40 | 72 | 14 | 4.72 | 4 | 5.27 | 14 |
| Poria monticola | 86 | 96 | 12 | 3.20 | 12 | 3.20 | 12 |
| Polyporus palustris | 91 | 94 | 8 | 1.55 | 8 | 1.55 | 8 |
| Lentinus lepideus | 20 | 23 | 20+ | 2.30 | 20 | 2.30 | 20 |
| Fomes subroseus | 77 | 83 | 10 | 4.00 | 10 | 4.01 | 10 |
| Lenzites trabea | 93 | 104 | 14 | 3.54 | 14 | 3.54 | 14 |
| Fomes roseus | 36 | 59 | 20+ | 3.48 | 18 | 3.50 | 20 |
| Fomes meliae | 106 | 112 | 8 | 2.12 | 12 | 2.64 | 8 |
| Fomes officinalis | 3 | 31 | 20+ | 4.52 | 20 | 4.52 | 20 |
| Poria xantha | 36 | 84 | 12 | 3.40 | 20 | 3.78 | 12 |
| Poria cocos | 30 | 40 | 20+ | 4.75 | 20 | 4.75 | 20 |
| Poria nigra | 147 | 156 | 14 | 2.30 | 14 | 2.30 | 14 |
| Poria vaillantii | No growth | | | | | | |
| Polyporus immitus | 18 | 75 | 20+ | 2.52 | 20+ | 2.52 | 20 |
| Poria oleraceae | 94 | 137 | 20+ | 2.03 | 20 | 2.03 | 20 |
| Poria luteofibrata | 5 | 36 | 40+ | 1.50 | 20 | 1.50 | 40 |
| Merulius lacrymans | 8 | 71 | 40+ | 2.90 | 12 | 2.95 | 40 |
| Polyporus sulphureus | 10 | 40 | 30+ | 5.15 | 20 | 5.40 | 30 |
| Lenzites striata | 10 | 42 | 14 | 4.20 | 12 | 4.26 | 14 |
| Coniophora cerebella | 24 | 32 | 14 | 4.25 | 14 | 4.25 | 14 |
| Trametes malicola | 38 | 60 | 10 | 2.89 | 14 | 3.21 | 10 |

+ maximum growth not yet reached.

TABLE 9 (CONTINUED)

COMPARISON OF SEVEN-DAY GROWTH AND MAXIMUM GROWTH IN GLUTAMIC ACID

Growth given as milligrams of dry weight of mycelium per 70 ml. of medium, averages of duplicate flasks. "Terminal" acidity means that at the time of maximum mycelial weight.

| Organism | Seven-day mycelial weight | Maximum mycelial weight | | Maximum acidity | | Terminal acidity | |
|---|---|---|---|---|---|---|---|
| Brown rots (con't) - | mg. | mg. | days | pH | days | pH | days |
| Polyporus spraguei | 32 | 46 | 16 | 3.60 | 16 | 3.60 | 16 |
| Polyporus betulinus | 67 | 92 | 12 | 2.17 | 14 | 2.26 | 12 |
| Polyporus schweinitzii | 37 | 48 | 16 | 4.16 | 16 | 4.16 | 16 |
| Lenzites saepiaria | 20 | 33 | 14 | 3.75 | 14 | 3.75 | 14 |
| Trametes serialis | 71 | 125 | 18 | 4.58 | 20 | 4.58 | 18 |
| Hydnum pulcherrimum | 24 | 42 | 18 | 4.90 | 18 | 4.90 | 18 |
| Ptychogaster rubescens | 122 | 132 | 14 | 5.03 | 12 | 5.10 | 14 |
| Daedalea quercina | 20 | 34 | 18 | 3.90 | 14 | 4.96 | 18 |
| White rots - | | | | | | | |
| Polyporus tulipiferus | 87 | 100 | 12 | 4.10 | 8 | 4.61 | 12 |
| Polyporus anceps | 59 | 111 | 20+ | 3.29 | 20 | 3.29 | 20 |
| Peniophora gigantea | 110 | 119 | 14 | 2.75 | 16 | 3.00 | 14 |
| Poria subacida | 59 | 68 | 12 | 4.01 | 12 | 4.01 | 12 |
| Fomes annosus | 70 | 83 | 12 | 3.95 | 12 | 3.95 | 12 |
| Lentinus tigrinus | 96 | 108 | 18 | 5.40 | 16 | 5.76 | 18 |
| Fomes fomentarius | 5 | 40 | 20 | 5.05 | 20 | 5.05 | 20 |
| Polyporus versicolor | 22 | 82 | 16 | 4.49 | 8 | 4.80 | 16 |
| Fomes geotropus | 59 | 129 | 16 | 4.40 | 10 | 4.99 | 16 |
| Polyporus abietinus | 24 | 170 | 18 | 5.20 | 12 | 5.49 | 18 |
| Fomes pini | 27 | 54 | 20+ | 4.54 | 14 | 4.63 | 20 |
| Polyporus fumosus | 18 | 37 | 16 | 1.64 | 16 | 1.65 | 16 |
| Armillaria mellea | 15 | 33 | 20+ | 4.57 | 10 | 4.90 | 20 |

+ maximum growth not yet reached.

The following generalizations and comments may be made regarding the data in Tables 8 and 9.

1. Maximum growth is attained in 8 to 20 ≠ days, with an average of 12.7 days in malt extract, and an average of 15.6 days in glutamic acid plus thiamine.

2. Except for Fomes annosus, the rate of growth of the fungi is greater in malt extract than in glutamic acid plus thiamine.

3. With two exceptions (Lenzites trabea and Ptychogaster rubescens) the maximum amount of growth attained is greater in malt extract than in glutamic acid plus thiamine.

4. Maximum acidity was attained in from 4 to 20 ≠ days, with an average of 14 days for the organisms in malt extract, and 14.6 days in glutamic acid. On the average then, in malt extract maximum growth is reached before maximum acidity, while in glutamic acid the opposite is true. There were, of course, certain specific exceptions to this latter statement.

5. Acid was invariably produced in both media (original pH 5.5): in malt extract (carbohydrate about 0.8%, mostly maltose), the maximum acidity varied from pH 1.55 to 4.55; in the glutamic acid medium (glucose 1%), from pH 1.55 to 5.40.

6. The lowest pH (1.5-2.0) was reached by Polyporus palustris in the malt extract and the glutamic acid, by Lentinus lepideus in malt extract, and by Poria vaillantii in malt extract. Very little acid (pH 5.0-5.5) was formed by Ptychogaster rubescens or Lentinus tigrinus in the glutamic acid medium.

7. The "terminal" pH values, recorded on the day that maximum growth was reached, show that in general there was a slight decrease in acidity from a maximum acidity previously reached.

## UTILIZATION OF DIFFERENT FORMS OF ORGANIC AND INORGANIC NITROGEN IN SYNTHETIC MEDIA

All of this work was done in standard shake culture, using controlled inoculum size, p H 5.0 - 5.5, etc., as described in the section on Methods. Because most of the wood rots grew well in shake culture in malt extract, a 1% solution of this material was used as a "control" against which growth in the known forms of nitrogen could be compared. Growth was determined quantitatively in the following nitrogen sources: 1% malt extract (control), vitamin-free casein hydrolysate, urea, ammonium carbonate, ammonium sulfate, ammonium nitrate, ammonium chloride, potassium nitrate, potassium nitrite, and 23 single amino acids and glutamine. Except for the malt extract control, the nitrogen source under test was added to the basal medium in Table 1. All tests were run both with and without added thiamine (1 mg. per liter), except that the malt extract, which contained a variety of vitamins, was used "as is". With the exception of two organisms, which will be considered later, growth did not take place in the absence of thiamine, and the summarized data (Tables 10 and 11) show only the results <u>with</u> thiamine except where otherwise indicated. The total nitrogen concentrations in the solutions of the various nitrogen sources were as follows: 1% malt extract, 0.004%; DL-mixtures of amino acids, 0.024%; ammonium nitrate, 0.012% as <u>ammonium</u> nitrogen; all others, 0.012%.

For convenience, growth weights in test nitrogen compounds are expressed as <u>percentages</u> of those obtained with the same organism in the standard 1% malt extract broth (control). These weights are an index of the relative utilisation of different nitrogen compounds by the fungi, but the fact that <u>some</u> growth is present in the third transfer does not necessarily mean that a given nitrogen compound will support growth "indefinitely" in continued subculture in that compound. Unless otherwise indicated (by a superscript) the data in Tables 10 and 11 refer to amounts of growth obtained in the third, serial, 7-day subculture in the test nutrient in question. The superscripts indicate the number of serial transfers to which the data applies (see next paragraph).

For those fungi which, after the third serial transfer in a test nitrogen source, produced less than 50% of the growth attained in the malt extract standard, serial subcultures were continued (maximum of nine) until growth either reached a constant amount or the organisms died out. Usually, three transfers were enough to rule out the "carry-over" of nutrients by an organism. Experiment also showed that if, in the third transfer in a given nitrogen source, the amount of growth was <u>more</u> than about 50% of that obtained in malt extract, the organism would continue to grow "indefinitely" upon continued serial transfer in that nutrient. When growth in the third transfer was <u>less</u> than about 50% of that in malt extract, it was found that while most organisms would continue to grow in further subculture, some eventually died out. Therefore, although <u>third-transfer</u> weights may be used <u>routinely</u> (i.e., as a preliminary screening) as an indication of the relative ability of a given organism to utilize a given nitrogen source, the third-transfer weights are not, in all cases, adequate criteria of <u>continued</u> growth in a given nutrient. Therefore, for each third-transfer weight which was <u>less</u> than 50% of the growth in malt extract for the same organism, we continued serial subculture until the growth either reached a constant level or the organism dies out. Another way of indicating this preliminary criterion of the nutritional adequacy of a nitrogen source for <u>continued</u> growth is to express it in terms of the amount of growth resulting from a given inoculum. The weight of our standard inoculum is 0.02-0.03 mg.

(for 70 ml. of culture medium), and the amount of growth in 7 days in malt extract averages about 100 mg., which is a 4000-fold increase in cell substance. By taking 50% of this as the point below which continued growth in subculture may in some cases be questionable, we are saying that when the increase in growth is less than 2000-fold, further subculture beyond three transfers is to be carried out. The amount of "carry-over" of nutrients by certain organisms may be greater than with others; also, slow-growing organisms will produce smaller amounts of growth in the standard 7-day incubation period.

Table 10 summarises comparative data on the growth of brown rots and white rots in a variety of organic and inorganic nitrogen compounds. Table 11 contains data on the same organisms in each of 23 amino acids and in glutamine. Except for malt extract, all nitrogen compounds were added to the basal medium (Table 1).

The Eh readings mentioned in the heading of Tables 10 and 11 are referable to the hydrogen electrode. (See later section for studies of Eh of cultures).

TABLE 10

GROWTH OF WOOD ROTS IN SHAKE CULTURE AT 28°C. WITH VARIOUS ORGANIC AND INORGANIC NITROGEN SOURCES

Growth in 1% malt extract (pH 4.9 (unadjusted), nitrogen=0.004%) given as milligrams of dry weight of mycelium per 70 ml. of medium (standard); growth in test media (70 ml., pH 5.0 - 5.5) expressed as percentages of that in the malt extract standard. Initial Eh = +400 to +500 m.v. Standard inoculum used. Test nitrogen compounds added to the basal medium (Table 1) to a total or, with ammonium nitrate, to an ammonium nitrogen concentration of 0.012%. All data are averages of at least duplicate, 7-day cultures, after at least three serial transfers in the given nutrient. A superscript after a figure shows the number of the serial transfer to which the data applies; unless otherwise indicated, figures are for the third subculture.

| Organism | Malt extract | Casein hydrolysate | L-Glutamic acid | Urea | $(NH_4)_2CO_3$ |
|---|---|---|---|---|---|
| Brown rots - | mg. | % | % | % | % |
| Poria incrassata | 80 | 68 | 49; $51^9$ | 25; $30^9$ | 30; $23^9$ |
| Poria monticola | 116 | 83 | 74 | 30; $26^9$ | 32; $25^9$ |
| Polyporus palustris | 111 | 57 | 82 | 25; $49^9$ | 47; $43^9$ |
| Lentinus lepideus | 80 | 130 | 25; $30^9$ | 0 | 74; $58^9$ |
| Fomes subroseus | 99 | 86 | 77 | 22; $54^9$ | 50; $43^9$ |
| Lenzites trabea | 54 | 71 | 169 | 30; $101^9$ | 42; $38^9$ |
| Fomes roseus | 106 | 99 | 33; $28^9$ | 21; $43^9$ | 40; $41^9$ |
| Fomes meliae | 125 | 79 | 83 | 20; $30^9$ | 29; $31^9$ |
| Fomes officinalis | 63 | 0 | 0 | 0 | 0 |
| Poria xantha | 72 | 96 | 118 | 48; $60^9$ | 58; $46^9$ |
| Poria cocos | 56 | 89 | 52; $48^9$ | 22; $42^9$ | 37; $40^9$ |
| Poria nigra | 147 | 85 | 97 | 29; $44^9$ | 41; $35^9$ |
| Poria vaillantii | 96 | 0 | 0 | 0 | 0 |
| Polyporus spraguei | 115 | 85 | 28; $30^9$ | 25; $37^9$ | 20; $26^9$ |
| Polyporus betulinus | 80 | 129 | 118 | 38; $69^9$ | 71; $56^9$ |
| Polyporus immitus | 88 | 22; $30^9$ | 29; $25^9$ | 14; $21^9$ | 36; $25^9$ |

(continued on next page)

TABLE 10 (CONTINUED)

| Organism | Malt extract | Casein hydrolysate | L-Glutamic acid | Urea | $(NH_4)_2CO_3$ |
|---|---|---|---|---|---|
| Brown rots (cont'd) – | mg. | % | % | % | % |
| Polyporus schweinitzii | 57 | 95 | 64 | 43; 62[9] | 49; 51[9] |
| Lenzites saepiaria | 83 | 52 | 24; 21[9] | 24; 52[9] | 51; 39[9] |
| Hydnum pulcherrimum | 82 | 94 | 48; 43[9] | 30; 28[9] | 15; 23[9] |
| Trametes serialis** | 221 | 95 | 49; 50[9] | 55; 56[9] | 39; 37[9] |
| Ptychogaster rubescens** | 89 | 137 | 149 | 40; 35[9] | 97; 73[9] |
| Daedalea quercina | 113 | 100 | 35; 35[9] | 34; 49[9] | 50; 43[9] |
| Poria oleraceae | 123 | 96 | 94 | 18; 28[9] | 26; 22[9] |
| Poria lutcofibrata | 10; 17[9] | 100; 0[4] | 50; 0[4] | 0 | 0 |
| Merulius lacrymans | 34; 29[9] | 59; 107[9] | 24; 33[9] | 21; 28[9] | 25; 28[9] |
| Polyporus sulphureus | 30; 43[9] | 127 | 30; 23[9] | 27; 23[9] | 33; 21[9] |
| Lenzites striata | 65; 53[9] | 66; 77[9] | 16; 32[9] | 19; 37[9] | 46; 52[9] |
| Coniophora cerebella | 65; 58[9] | 89 | 38; 55[9] | 35; 44[9] | 37; 36[9] |
| Trametes malicola | 65 | 61; 64[9] | 66; 73[9] | 61; 63[9] | 44; 54[9] |
| White rots – | | | | | |
| Polyporus tulipiferus | 130 | 50; 52[9] | 66 | 15; 47[9] | 45; 41[9] |
| Polyporus anceps | 102 | 96 | 57; 53[9] | 15; 34[9] | 39; 26[9] |
| Peniophora gigantea | 97 | 136 | 112 | 36; 47[9] | 46; 40[9] |
| Poria subacida | 97 | 62 | 61 | 7; 16[9] | 56; 50[9] |
| Fomes fomentarius | 71 | 74 | 26; 23[9] | 23; 60[9] | 21; 64[9] |
| Polyporus versicolor | 120 | 75 | 80 | 28; 20[9] | 21; 21[9] |
| Fomes annosus | 94 | 92 | 73 | 8; 21[9] | 5; 12[9] |
| Lentinus tigrinus | 100 | 97 | 96 | 11; 25[9] | 22; 20[9] |
| Fomes geotropus | 123 | 99 | 47; 50[9] | 18; 25[9] | 28; 32[9] |
| Polyporus abietinus | 153 | 106 | 98 | 95; 50[9] | 32; 30[9] |

** Will grow without thiamin in the media shown.

(continued on next page)

TABLE 10 (CONTINUED)

| Organism | Malt extract | Casein hydrolysate | L-Glutamic acid | Urea | $(NH_4)_2CO_3$ |
|---|---|---|---|---|---|
| White rots (cont'd) - | mg. | % | % | % | % |
| Fomes pini | 68 | 63; 84[9] | 44; 70[9] | 44; 58[9] | 47; 48[9] |
| Polyporus fumosus | 69 | 47; 59[9] | 30; 43[9] | 17; 57[9] | 33; 36[9] |
| Armillaria mellea | 11;21[9] | 87; 57[9] | 0(120)* | 0 | 0 |

* After "training" (continued subculture).

(continued on next page)

TABLE 1C (CONTINUED)

| Organism | $(NH_4)_2SO_4$ | $NH_4NO_3$ | $NH_4Cl$ | $KNO_3$ | $KNO_2$ |
|---|---|---|---|---|---|
| Brown rots - | % | % | % | % | % |
| Poria incrassata | $25; 22^9$ | $42; 51^9$ | 0 | 0 | 0 |
| Poria monticola | $26; 27^9$ | $32; 31^9$ | $8; 0^4$ | 0 | 0 |
| Polyporus palustris | $42; 45^9$ | $42; 45^9$ | $9; 0^4$ | 0 | 0 |
| Lentinus lepideus | $60; 74^9$ | $93; 98^9$ | $20; 0^4$ | 0 | 0 |
| Fomes subroseus | $38; 37^9$ | $51; 42^9$ | $12; 0^4$ | 0 | 0 |
| Lenzites trabea | $24; 26^9$ | $23; 32^9$ | $6; 0^4$ | 0 | 0 |
| Fomes roseus | $33; 29^9$ | $40; 38^9$ | $2; 0^4$ | 0 | 0 |
| Fomes meliae | $31; 25^9$ | $33; 28^9$ | $6; 0^4$ | 0 | 0 |
| Fomes officinalis | 0 | $19; 0^4$ | 0 | 0 | 0 |
| Poria xantha | $46; 47^9$ | $79; 76^9$ | $8; 0^4$ | 0 | 0 |
| Poria cocos | $24; 19^9$ | $54; 63^9$ | 0 | 0 | 0 |
| Poria nigra | $30; 27^9$ | $54; 49^9$ | $7; 0^4$ | 0 | 0 |
| Poria vaillantii | 0 | 0 | 0 | 0 | 0 |
| Polyporus spraguei | $19; 20^9$ | $27; 27^9$ | $3; 0^4$ | 0 | 0 |
| Polyporus betulinus | $53; 51^9$ | $73; 65^9$ | $9; 0^4$ | 0 | 0 |
| Polyporus immitus | $30; 25^9$ | $48; 48^9$ | 0 | 0 | 0 |
| Polyporus schweinitzii | $41; 41^9$ | $57; 55^9$ | $9; 0^4$ | 0 | 0 |
| Lenzites saepiaria | $43; 37^9$ | $59; 55^9$ | $11; 0^4$ | 0 | 0 |
| Hydnum pulcherrimum | $12; 26^9$ | $30; 34^9$ | $4; 0^4$ | 0 | 0 |
| Trametes serialis ** | $32; 30^9$ | $44; 39^9$ | $37; 33^9$ | $22; 0^6$ | 0 |
| Ptychogaster rubescens** | $72; 79^9$ | $96; 79^9$ | $25; 0^6$ | $25; 0^7$ | $22; 0^6$ |
| Daedalea quercina | $36; 38^9$ | $59; 52^9$ | 0 | 0 | 0 |
| Poria oleraceae | $21; 18^9$ | $33; 29^9$ | $6; 0^4$ | 0 | 0 |
| Poria luteofibrata | 0 | 0 | 0 | 0 | 0 |

** All grown without thiamin in the media shown.

(continued on next page)

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TABLE 10 (CONCLUDED)

| Organism | $(NH_4)_2SO_4$ | $NH_4NO_3$ | $NH_4Cl$ | $KNO_3$ | $KNO_2$ |
|---|---|---|---|---|---|
| Brown rots (cont'd) - | % | % | % | % | % |
| Merulius lacrymans | $16;\ 49^{9}$ | $29;\ 53^{9}$ | 0 | 0 | 0 |
| Polyporus sulphureus | $37;\ 17^{9}$ | $43;\ 30^{9}$ | 0 | 0 | 0 |
| Lenzites striata | $37;\ 44^{9}$ | $47;\ 75^{9}$ | 0 | $14;0^{5}$ | $18;0^{4}$ |
| Coniophora cerebella | $30;\ 32^{9}$ | $46;\ 47^{9}$ | 0 | 0 | 0 |
| Trametes malicola | $41;\ 42^{9}$ | $62;\ 72^{9}$ | 0 | 0 | 0 |
| White rots - | | | | | |
| Polyporus tulipiferus | $33;\ 38^{9}$ | $49;\ 47^{9}$ | $3;\ 0^{4}$ | 0 | 0 |
| Polyporus anceps | $28;\ 21^{9}$ | $46;\ 41^{9}$ | $8;\ 0^{4}$ | 0 | 0 |
| Peniophora gigantea | $34;\ 29^{9}$ | $48;\ 44^{9}$ | $9;\ 0^{4}$ | 0 | 0 |
| Poria subacida | $44;\ 41^{9}$ | $55;\ 52^{9}$ | 0 | 0 | 0 |
| Fomes fomentarius | $28;\ 36^{9}$ | $37;\ 35^{9}$ | $3;\ 0^{4}$ | 0 | 0 |
| Polyporus versicolor | $23;\ 21^{9}$ | $25;\ 23^{9}$ | $8;\ 0^{4}$ | 0 | 0 |
| Fomes annosus | $5;\ 6^{9}$ | $63;\ 51^{9}$ | 0 | 0 | 0 |
| Lentinus tigrinus | $13;\ 16^{9}$ | $32;\ 32^{9}$ | $5;\ 0^{4}$ | 0 | 0 |
| Fomes geotropus | $21;\ 23^{9}$ | $43;\ 41^{9}$ | $3;\ 0^{4}$ | 0 | 0 |
| Polyporus abietinus | $21;\ 22^{9}$ | $35;\ 36^{9}$ | $8;\ 0^{4}$ | 0 | 0 |
| Fomes pini | $42;\ 44^{9}$ | $51;\ 56^{9}$ | $13;\ 0^{4}$ | 0 | 0 |
| Polyporus fumosus | $30;\ 36^{9}$ | $44;\ 51^{9}$ | 0 | $26;0^{7}$ | $20;0^{6}$ |
| Armillaria mellea | 0 | 0 | 0 | 0 | 0 |

TABLE 11

GROWTH OF WOOD ROTS IN SHAKE CULTURE AT 28°C. IN SINGLE AMINO ACIDS COMPARED WITH CERTAIN OTHER NITROGEN COMPOUNDS

Growth in 1% malt extract (pH 4.9 (unadjusted), nitrogen = 0.004%) given as milligrams of dry weight of mycelium per 70 ml. of medium (standard); growth in test media (70 ml., pH 5.0-5.5) expressed as percentages of that in the malt extract standard. Initial Eh = +400 to +500 m.v., except L-cysteine Eh = +100 m.v. Standard inoculum used. Test nitrogen compounds added to the basal medium (Table 1) to a total nitrogen concentration of 0.012%, except DL-mixtures of amino acids to 0.024%. All data are averages of at least duplicate, 7-day cultures, after at least three serial transfers in the given nutrient. A superscript after a figure shows the number of the serial transfer to which the data applies; unless otherwise indicated, figures are for the third subculture.

| Organism | Malt extract | Casein hydro-lysate | $(NH_4)_2CO_3$ | Glycine | B-alanine | DL-valine |
|---|---|---|---|---|---|---|
| Brown rots - | mg. | % | % | % | % | % |
| Daedalea quercina | 110 | 100 | 50; $43^9$ | 19; $25^9$ | 46; $41^9$ | 82 |
| Fomes subroseus | 94 | 86 | 52 | 77 | 37; $31^9$ | 91 |
| Hydnum pulcherrimum | 73 | 95 | 15; $23^9$ | 75 | 33; $42^9$ | 55 |
| Lentinus lepideus | 83 | 131 | 78 | 35; $37^9$ | 44; $44^9$ | 54; $57^9$ |
| Lenzites trabea | 61 | 72 | 42; $38^9$ | 145 | 48; $54^9$ | 170 |
| Polyporus palustris | 103 | 57 | 50 | 78 | 47; $39^9$ | 94 |
| Poria monticola | 113 | 82 | 32; $25^9$ | 73 | 23; $24^9$ | 66 |
| Ptychogaster rubescens** | 97 | 137 | 90 | 108 | 16; $34^9$ | 208 |
| Trametes serialis** | 126 | 95 | 38; $34^9$ | 40; $43^9$ | 10; $16^9$ | 54; $48^9$ |
| White rots - | | | | | | |
| Fomes annosus | 82 | 92 | 5; $12^9$ | 80 | 6; $8^9$ | 125 |
| Lentinus tigrinus | 106 | 97 | 22; $20^9$ | 85 | 38; $33^9$ | 96 |
| Peniophora gigantea | 104 | 136 | 46; $40^9$ | 108 | 12; $18^9$ | 94 |
| Polyporus anceps | 93 | 96 | 30; $26^9$ | 69 | 33; $28^9$ | 76 |
| Polyporus tulipiferus | 120 | 50 | 45; $41^9$ | 66 | 17; $21^9$ | 78 |
| Poria subacida | 89 | 62 | 56 | 55 | 23; $29^9$ | 59 |

** Will grow without thiamine in the media shown.

(continued on next page)

TABLE 11 (CONTINUED)

| Organism | L-leucine | DL-iso-leucine | DL-serine | DL-threo-nine | L-cys-teine* | L-cys-tine |
|---|---|---|---|---|---|---|
| Brown rots - | % | % | % | % | % | % |
| Daedalea quercina | 17; 22[9] | 26; 19[9] | 24; 26[9] | 22; 24[9] | 17; 0[5] | 52 |
| Fomes subroseus | 52 | 43; 39[9] | 56 | 59 | 0 | 45; 38[9] |
| Hydnum pulcherrimum | 62 | 58 | 65 | 67 | 14; 0[4] | 44; 37[9] |
| Lentinus lepideus | 15; 24[9] | 12; 24[9] | 24; 17[9] | 19; 20[9] | 0 | 14; 11[9] |
| Lenzites trabea | 113 | 99 | 118 | 119 | 30; 0[5] | 58 |
| Polyporus palustris | 49; 53[9] | 50; 46[9] | 64 | 66 | 15; 0[4] | 50 |
| Poria monticola | 49; 45[9] | 32; 28[9] | 56 | 56 | 18; 0[5] | 18; 22[9] |
| Ptychogaster rubescens** | 105 | 92 | 104 | 90 | 9; 0[4] | 6; 11[9] |
| Trametes serialis** | 42; 43[9] | 25; 32[9] | 31; 34[9] | 29; 25[9] | 19; 0[5] | 16; 13[9] |
| White rots - | | | | | | |
| Fomes annosus | 51 | 51; 45[9] | 54 | 52 | 5; 0[4] | 4; 7[9] |
| Lentinus tigrinus | 66 | 54 | 70 | 73 | 15; 0[5] | 55 |
| Peniophora gigantea | 78 | 70 | 85 | 85 | 10; 0[4] | 13; 9[9] |
| Polyporus anceps | 48; 40[9] | 39; 32[9] | 53 | 52 | 11; 0[4] | 73 |
| Polyporus tulipiferus | 48; 39[9] | 39; 34[9] | 57 | 55 | 15; 0[5] | 27; 32[9] |
| Poria subacida | 34; 27[9] | 34; 29[9] | 28; 32[9] | 39; 35[9] | 5; 0[4] | 33; 23[9] |

* Eh of medium as used = + 300 m.v.; L-cysteine utilised by all organisms at Eh of + 400 to + 500 m.v.

** Will grow without thiamine in the media shown.

(continued on next page)

TABLE 11 (CONTINUED)

| Organism | DL-methionine | DL-aspartic acid | L-glutamic acid | DL-phenylalanine | L-tyrosine |
|---|---|---|---|---|---|
| Brown rots - | % | % | % | % | % |
| Daedalea quercina | 20; 29[9] | 28; 23[9] | 35; 35[9] | 32; 29[9] | 28; 23[9] |
| Fomes subroseus | 45; 30[9] | 77 | 79 | 54 | 43; 33[9] |
| Hydnum pulcherrimum | 61 | 77 | 48; 43[9] | 63 | 39; 35[9] |
| Lentinus lepideus | 15; 13[9] | 40; 29[9] | 25; 30[9] | 11; 19[9] | 15; 10[9] |
| Lenzites trabea | 18; 10[9] | 147 | 148 | 107 | 87 |
| Polyporus palustris | 61 | 80 | 87 | 55; 49[9] | 41; 44[9] |
| Poria monticola | 53 | 76 | 75 | 48; 52[9] | 30; 36[9] |
| Ptychogaster rubescens** | 103 | 117 | 123 | 99 | 52; 62[9] |
| Trametes serialis** | 29; 40[9] | 30; 24[9] | 49; 50[9] | 29; 21[9] | 24; 18[9] |
| White rots - | | | | | |
| Fomes annosus | 45; 40[9] | 83 | 85 | 50; 45[9] | 37; 47[9] |
| Lentinus tigrinus | 56 | 88 | 94 | 61 | 51 |
| Peniophora gigantea | 84 | 111 | 110 | 78 | 48; 41[9] |
| Polyporus anceps | 50; 40[9] | 73 | 65 | 44; 38[9] | 22; 32[9] |
| Polyporus tulipiferus | 51; 64[9] | 72 | 75 | 45; 50[9] | 30; 32[9] |
| Poria subacida | 39; 30[9] | 57 | 58 | 28; 32[9] | 20; 28[9] |

** Will grow without thiamine in the media shown.

(continued on next page)

TABLE 11 (CONTINUED)

| Organism | L-trypto-phane | L-proline | L-hydroxy-proline | L-histi-dine | DL-nor-leucine |
|---|---|---|---|---|---|
| Brown rots - | % | % | % | % | % |
| Daedalea quercina | 18; 22[9] | 22; 21[9] | 28; 24[9] | 32; 36[9] | 30; 20[9] |
| Fomes subroseus | 60 | 74 | 63; 54[9] | 45;36[9] | 43;52[9] |
| Hydnum pulcherrimum | 65 | 80 | 56; 62[9] | 42;45[9] | 30; 38[9] |
| Lentinus lepideus | 19; 24[9] | 39; 35[9] | 41; 37[9] | 13; 12[9] | 19; 14[9] |
| Lenzites trabea | 120 | 137 | 140 | 83 | 78 |
| Polyporus palustris | 65 | 81 | 78 | 38; 43[9] | 46;63[9] |
| Poria monticola | 57 | 73 | 57; 50[9] | 45; 35[9] | 39;27[9] |
| Ptychogaster rubescens** | 100 | 107 | 107 | 54 | 52 |
| Trametes serialis** | 28; 25[9] | 36; 45[9] | 32; 37[9] | 24; 30[9] | 17; 24[9] |
| White rots - | | | | | |
| Fomes annosus | 53 | 74 | 77 | 40;32[9] | 36;42[9] |
| Lentinus tigrinus | 74 | 85 | 47; 37[9] | 47;68[9] | 44;39[9] |
| Peniophora gigantea | 86 | 106 | 69 | 47;51[9] | 42;39[9] |
| Polyporus anceps | 54 | 69 | 51; 43[9] | 31; 40[9] | 39; 42[9] |
| Polyporus tulipiferus | 54 | 70 | 34; 29[9] | 38; 46[9] | 35; 50[9] |
| Poria subacida | 38;22[9] | 52 | 69 | 23; 37[9] | 28; 21[9] |

** Will grow without thiamine in the media shown.

(continued on next page)

TABLE 11 (CONCLUDED)

| Organism | L-asparagine | Glutamine | DL-ornithine | L-lysine | L-arginine |
|---|---|---|---|---|---|
| Brown rots - | % | % | % | % | % |
| Daedalea quercina | 21; 27[9] | 24; 27[9] | 18; 24[9] | 23; 27[9] | 20; 26[9] |
| Fomes subroseus | 76 | 85; 89[9] | 72 | 37; 30[9] | 78 |
| Hydnum pulcherrimum | 85 | 33; 40[9] | 79 | 26; 32[9] | 85 |
| Lentinus lepideus | 50 | 49; 57[9] | 20; 29[9] | 18; 24[9] | 36; 25[9] |
| Lenzites trabea | 142 | 150 | 134 | 50 | 145 |
| Polyporus palustris | 87 | 88 | 78 | 30; 25[9] | 79 |
| Poria monticola | 77 | 80; 90[9] | 72 | 28; 31[9] | 73 |
| Ptychogaster rubescens** | 98 | 122 | 114 | 31; 39[9] | 100 |
| Trametes serialis** | 39; 35[9] | 48; 52[9] | 25; 32[9] | 20; 22[9] | 40; 37[9] |
| White rots - | | | | | |
| Fomes annosus | 83 | 85 | 72 | 34; 37[9] | 83 |
| Lentinus tigrinus | 87 | 106 | 79 | 32; 32[9] | 87 |
| Peniophora gigantea | 106 | 129 | 99 | 21; 26[9] | 109 |
| Polyporus anceps | 67 | 71 | 65 | 20; 16[9] | 72 |
| Polyporus tulipiferus | 72 | 85 | 66 | 23; 23[9] | 69 |
| Poria subacida | 55 | 81 | 51 | 21; 30[9] | 62; 59[9] |

** Will grow without thiamine in the media shown..

From Table 10 certain important generalizations and specific conclusions can be drawn, as follows:

1. Malt extract supports continued growth of all the organisms, although *Merulius lacrymans*, *Polyporus sulphureus*, *Poria luteofibrata*, and *Armillaria mellea* produce only small amounts of mycelium in 7 days compared with rest of the fungi. The growth curve data for these species show them to be slow-growing organisms, (see Table 8, above), which probably explains their apparently poor growth in 7 days in malt extract.

2. Of the organic nitrogen sources, growth of all organisms in urea was fairly uniformly poor, and in most cases was less than in ammonium carbonate, ammonium sulfate and ammonium nitrate. It is of interest that most organisms which grow in urea show a marked increase in the amount of growth from the third to the ninth serial subculture.

3. Ammonium carbonate, ammonium sulfate and ammonium nitrate are roughly equivalent in their growth-supporting ability.

4. Generally speaking, the nitrogen sources in the basal medium support growth in the presence of thiamine but not in its absence. This indicates that for most of the fungi thiamine is necessary and is the only essential vitamin. The exceptions to this generalization will be mentioned below.

5. *Poria vaillantii*, *Fomes officinalis* and *Poria luteofibrata* grew only in malt extract: none of the other nitrogen compounds, organic or inorganic, plus thiamine, supported growth in continued subculture from malt extract. This suggests a vitamin definiency in the synthetic media for these organism. *Armillaria mellea* grew only in casein hydrolysate and (eventually) glutamic acid in addition to the malt extract. (See later for discussion of special growth conditions for these 4 organisms.).

(See

6. Ammonium chloride supported growth of _Trametes serialis_ only, under the conditions used. (See below for the effect of succinic acid on growth in ammonium carbonate).

7. Potassium nitrate and nitrite did not support continued growth of any species.

8. Both _Trametes serialis_ and _Ptychogaster rubescens_ grew in their various nitrogen sources in the complete absence of thiamine, although the growth weights attained were nearly all (exception: _P. rubescens_ in $NH_4NO_3$) less than 50% of those in malt extract, and were always less than the corresponding weights in the presence of thiamine. These figures indicate ability to grow without thiamine, although at a slower rate.

9. Some organisms grew outstandingly better in certain nitrogen sources than they did in malt extract: for example, _Lenzites trabea_ in glutamic acid; _Lentinus lepideus_ in casein hydrolysate; _Polyporus betulinus_ in casein hydrolysate and in glutamic acid; _Ptychogaster rubescens_ in casein hydrolysate and in glutamic acid; _Polyporus sulphureus_ in casein hydrolysate, and _Peniophora gigantea_ in casein hydrolysate.

3

From Table 11 the following generalisations and conclusions may be drawn for the organisms tabulated therein.

1. Generally, each of the amino acids singly in the basal medium was able to support growth of the organisms in continued subculture "indefinitely", although there were wide quantitative differences in the amount of growth so obtained. Exception: L-cysteine (see section below on Eh).

2. As among the various species of fungi, Trametes serialis, Lentinus lepideus and Daedalea quercina grew most poorly in the amino acids.

3. The best growth in single amino acids (with thiamine) was obtained with Lenzites trabea and Ptychogaster rubescens: in certain amino acids 1½ to 2 times as much growth was attained as in malt extract. For example, Lenzites trabea in glycine, aspartic acid, glutamic acid, proline, hydroxyproline, asparagine, glutamine, ornithine and arginine; Ptychogaster rubescens in valine and in glutamic acid.

4. Only Trametes serialis and Ptychogaster rubescens grew in the ~~delete~~ continued transfer in each of the amino acids without thiamine, although the amount of growth was less than a 2000-fold increase and was invariably less than in the presence of thiamine.

Miscellaneous studies on nitrogen compounds.

As regards the additive effect of amino acids, a limited amount of work with Polyporus palustris indicates that in general optimum growth is obtained with a mixture of several amino acids rather than any one. For example, if to the basal medium plus glutamic acid is added aspartic acid and/or valine and/or arginine and/or proline, there is a successive increase in the resultant growth for each additional amino acid used, within limits, even though the total nitrogen in each medium is the same. This observation suggests why, in general, more growth is obtained in the mixture of amino acids present in casein hydrolysate that in single amino acids. (Tables 10 and 11) We have not determined what is a full complement of amino acids even for Polyporus palustris.

Attempts were made to grow P. palustris and P. tuliniferus in nitrate and nitrite. Because none of the fungi grew in the basal medium with thiamine and nitrate or nitrite (Table 10), it was thought possible that other vitamins were needed for growth. P. palustris and P. tulipiferus were investigated in this regard, using sodium nitrate and sodium nitrite separately in the basal medium with thiamine (1 mg./liter), and adding the following nutrilites in various combinations: riboflavin, pyridoxine, pantothenic acid, niacin - 1 mg. per liter; biotin, folic acid - 2 gammas per liter; guanine, adenine, uracil - 13 mg. per liter; guanylic acid, adenylic acid, sodium nucleate, xanthine - 20 mg. per liter; choline, inositol - 2 mg. per liter.

In neither nitrogen compound with any combination of nutrilites was there growth even in the second transfer, and often not in the first transfer from malt extract. Assuming that the total concentration of nitrogen was not too high to be inhibitory (120 mg. N per liter for nitrate or nitrite, plus as much as 65 mg. N per liter for the nutrilites), it seems clear that these organisms actually lack a biochemical mechanism for utilization of nitrogen in the form of nitrate or nitrite.

With the exception of Trametes serialis, none of the wood rots grew in continued subculture in the basal medium with ammonium chloride as the source of nitrogen (Table 10). It was found by chance that when a trace of succinic acid was added to some of the cultures in the ammonium chloride medium, growth took place. The following have been found to grow in continued serial subculture (6) in the basal medium (Table 1) plus ammonium chloride (0.012% nitrogen) plus succinic acid

(10 mg. per liter): Poria luteofibrata, Polyporus palustris, Lenzites trabea, Trametes serialis, Poria monticola, Lentinus tigrinus, Polyporus anceps, Ptychogaster rubescens, Peniophora gigantea, Hydnum pulcherrimum, Lentinus lepideus, and Poria subacida. The following organisms would not continue to grow in the indicated medium: Fomes annosus, Daedalea quercina, and Fomes subroseus. They died out in the 4th, 2nd and 3rd serial transfers, respectively. Growth in other inorganic nitrogen sources, such as ammonium sulfate, ammonium carbonate and ammonium nitrate, was accelerated or increased in total amount by the addition of succinic acid. Explanation of this phenomenon may be the initiation or acceleration of the Krebs cycle by the succinic acid. As a matter of interest, following the effect of succinic acid on growth in ammonium chloride, the effect of succinic acid was tried in the basal medium plus ammonium carbonate, in which some growth of nearly all organisms had previously been observed. Data for several species are shown in Table 12. It is evident that there is some growth stimulation in all cases. Succinic acid increases the growth of Fomes annosus some 11-fold, of Lentinus tigrinus nearly 4-fold, and doubles the growth of Peniophora gigantea.

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TABLE 12

EFFECT OF SUCCINIC ACID ON GROWTH OF CERTAIN WOOD-ROTS IN SYNTHETIC MEDIUM WITH AMMONIUM CARBONATE

Basal medium plus ammonium carbonate (0.012% nitrogen) plus succinic acid (10 mg. per liter). pH 5.0 - 5.5. Growth in 1% malt extract (pH 4.9, nitrogen ± 0.004%) given as milligrams of dry weight of mycelium per 70 ml. of medium; growth in test medium (70 ml.) expressed as percentages of that in the malt extract. All data are averages of duplicate, 7-day, third-transfer cultures. at 28°C.

| Organism | Malt extract | Basal medium + $(NH_4)_2CO_3$ | Basal medium + $(NH_4)_2CO_3$ + succinic acid |
|---|---|---|---|
| Brown rots - | mg. | % | % |
| Polyporus palustris | 110 | 45 | 57 |
| Lenzites trabea | 62 | 39 | 48 |
| Trametes serialis A | 120 | 42 | 56 |
| Poria monticola | 110 | 30 | 45 |
| Ptychogaster rubescens | 87 | 86 | 102 |
| White rots - | | | |
| Polyporus tulipiferus | 125 | 42 | 59 |
| Fomes annosus | 95 | 5 | 66 |
| Lentinus tigrinus | 95 | 24 | 80 |
| Polyporus anceps | 111 | 32 | 47 |
| Peniophora gigantea | 95 | 44 | 85 |

Utilisation of amino acids as carbon sources was studied with two fungi, *P. palustris* (brown rot) and *P. tulipiferus* (white rot). These were tested for growth in shake culture in the basal medium minus glucose, but with various single amino acids as the sole source of nitrogen and carbon. Growth in continued subculture did not occur in any of the amino acids tested, viz: alpha alanine, beta alanine, l-arginine, l-asparagine, dl-aspartic acid, l-cystine, l-glutamic acid, glycine, l-histidine, dl-isoleucine, dl-leucine, l-lysine, dl-methionine, d-norleucine, d-ornithine, dl-phenylalanine, dl-serine, l-tryptophane, dl-valine.

Utilisation of l-glutamic acid as a nitrogen and carbon source by all 43 of our fungi also was investigated. All of the cultures were grown in shake culture in the basal medium minus glucose, but with l-glutamic acid (carbon concentration equivalent to that in 1% glucose solution) as the sole source of carbon and nitrogen. None of the species grew in continued subculture in this medium.

To determine the utilization of L- and DL- forms of amino acids, each of ten organisms was cultured in the basal medium with L- and DL-tryptophane and with L- and DL-leucine at various levels as the only source of nitrogen. The results (Table 13) indicate that only the natural (L- form) of the amino acids is utilised, a not unexpected finding. (It was for this reason that racemic mixtures (DL-) of amino acids were always used in a total nitrogen concentration (0.024%) twice that of other nitrogen compounds (0.012%) in Tables 10 and 11). If only the L- form of the acid is utilized, the amounts of growth for each organism in Table 13 should be the same for the L- and the DL- forms, since the latter were used in double the concentration of the former. This is seen to be the case throughout, although at the highest nitrogen levels (above optimum concentration) slight deviations are apparent.

# TABLE 13

## UTILIZATION OF L- AND DL- FORMS OF AMINO ACIDS

Based on dry weight of mycelium produced in 70 ml. of basal medium (Table 1) plus the indicated nitrogen source. Initial pH approximately optimum for each species. Concentrations of amino acids expressed as percentages of amino nitrogen. DL- amino acids used in double the concentrations of the L-forms. Standard inoculum used. Data are for duplicate, 7-day, third-transfer cultures.

### L- and DL- tryptophane

| % amino nitrogen of | | Mycelial weight-milligrams | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | P.tulipiferus | | P.palustris | | L. trabea | | F.annosus | | T.serialis | |
| L- acid | DL- acid | L- | DL- | L- | DL- | L- | DL- | L- | DL- | L- | DL- |
| | | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| 0.0004 | 0.0008 | 18 | 10 | 5 | 7 | 18 | 18 | 2 | 5 | 11 | 12 |
| 0.0008 | 0.0016 | 17 | 18 | 18 | 20 | 21 | 22 | 8 | 10 | 14 | 15 |
| 0.0015 | 0.0030 | 24 | 24 | 27 | 27 | 32 | 33 | 12 | 12 | 19 | 22 |
| 0.0030 | 0.0060 | 31 | 35 | 40 | 38 | 38 | 39 | 16 | 17 | 20 | 24 |
| 0.0060 | 0.0120 | 54 | 56 | 52 | 54 | 54 | 57 | 32 | 35 | 30 | 32 |
| 0.0120 | 0.0240 | 60 | 62 | 65 | 62 | 70 | 73 | 40 | 41 | 32 | 35 |
| 0.0240 | 0.0480 | 79 | 87 | 92 | 122 | 84 | 89 | 49 | 56 | 58 | 64 |

| % amino nitrogen of | | Mycelial weight-milligrams | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | P. monticola | | L.tigrinus | | P. anceps | | P.rubescens | | P.gigantea | |
| L- acid | DL- acid | L- | DL- | L- | DL- | L- | DL- | L- | DL- | L- | DL- |
| | | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| 0.0004 | 0.0008 | 18 | 16 | 15 | 15 | 16 | 17 | 30 | 28 | 24 | 26 |
| 0.0008 | 0.0016 | 20 | 18 | 30 | 32 | 24 | 25 | 51 | 58 | 38 | 39 |
| 0.0015 | 0.0030 | 20 | 22 | 42 | 44 | 25 | 28 | 62 | 60 | 40 | 42 |
| 0.0030 | 0.0060 | 38 | 38 | 60 | 59 | 32 | 32 | 76 | 77 | 52 | 52 |
| 0.0060 | 0.0120 | 42 | 43 | 65 | 67 | 41 | 45 | 84 | 87 | 70 | 71 |
| 0.0120 | 0.0240 | 65 | 69 | 78 | 79 | 50 | 52 | 95 | 98 | 89 | 99 |
| 0.0240 | 0.0480 | 82 | 90 | 88 | 100 | 59 | 71 | 132 | 176 | 111 | 128 |

(continued on next page)

TABLE 13 (CONCLUDED)

**L- and DL- leucine**

| % amino nitrogen of L- acid | % amino nitrogen of DL- acid | P.tulipiferus L- mg. | P.tulipiferus DL- mg. | P.palustris L- mg. | P.palustris DL- mg. | L. trabea L- mg. | L. trabea DL- mg. | F.annosus L- mg. | F.annosus DL- mg. | T.serialis L- mg. | T.serialis DL- mg. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.0004 | 0.0008 | 8 | 8 | 10 | 12 | 14 | 15 | 3 | 5 | 0 | 0 |
| 0.0008 | 0.0016 | 16 | 17 | 17 | 18 | 21 | 22 | 8 | 10 | 8 | 10 |
| 0.0015 | 0.0030 | 27 | 28 | 22 | 22 | 30 | 32 | 16 | 18 | 16 | 17 |
| 0.0030 | 0.0060 | 38 | 40 | 31 | 30 | 38 | 38 | 27 | 29 | 20 | 22 |
| 0.0060 | 0.0120 | 50 | 54 | 44 | 45 | 44 | 45 | 38 | 37 | 24 | 25 |
| 0.0120 | 0.0240 | 62 | 70 | 53 | 60 | 68 | 69 | 43 | 50 | 28 | 30 |
| 0.0240 | 0.0480 | 63 | 92 | 82 | 110 | 92 | 94 | 59 | 74 | 49 | 56 |

| % amino nitrogen of L- acid | % amino nitrogen of DL- acid | P. [illegible] L- mg. | P. [illegible] DL- mg. | L.tigrinus L- mg. | L.tigrinus DL- mg. | P. anceps L- mg. | P. anceps DL- mg. | P.rubescens L- mg. | P.rubescens DL- mg. | P.gigantea L- mg. | P.gigantea DL- mg. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.0004 | 0.0008 | 12 | 13 | 12 | 13 | 0 | 0 | 10 | 12 | 20 | 22 |
| 0.0008 | 0.0016 | 24 | 25 | 16 | 16 | 8 | 10 | 20 | 22 | 38 | 40 |
| 0.0015 | 0.0030 | 31 | 32 | 28 | 30 | 16 | 15 | 38 | 4[illegible] | 54 | 52 |
| 0.0030 | 0.0060 | 40 | 44 | 4[illegible] | 44 | 22 | 21 | 41 | 43 | 62 | 6[illegible] |
| 0.0060 | 0.0120 | 48 | 50 | 58 | 57 | 38 | 40 | 72 | 74 | 70 | 68 |
| 0.0120 | 0.0240 | 58 | 62 | 68 | 72 | 43 | 45 | 98 | 110 | 75 | 79 |
| 0.0240 | 0.0480 | 73 | 85 | 98 | 120 | 64 | 72 | 183 | 199 | 96 | 122 |

## OXIDATION-REDUCTION POTENTIAL (Eh) STUDIES OF SHAKE CULTURES

Although it was known that the wood rots were aerobic rather than anaerobic, and grew well in ordinary oxidised media (+ Eh) exposed to the air, nothing was known of their actual relations to redox potential in shake culture. In this regard, then, determinations were made of the Eh of all media before inoculation, and a study also was carried out of the Eh changes in shake culture during growth. Eh readings, particularly in cultures exposed to the air, are necessarily inconstant, even varying widely among replicate flasks, so that the data are indicative of typical readings rather than showing accurate values. Further, as is well known, Eh varies with pH, and since pH was not constant under various cultural conditions, the Eh values are not strictly comparable on a constant pH basis.

Table 14 shows typical initial Eh and pH readings before inoculation for most of the media used in Tables 10 and 11. The media were autoclaved, agitated on the shaker for one hour, then allowed to stand for one-half hour before Eh readings were made. This procedure simulated the conditions preceding the inoculation of a flask in routine nutrition studies. Examination of Table 14 shows that all media except that containing L-cysteine had relatively high oxidation-reduction potentials, the Eh in most cases being in the range of + 400 to + 500 millivolts (m.v.). L-cysteine, a strong reducing agent, gave a relatively low Eh of about + 350 m.v. It is evident that the media in which our fungi grow well are quite highly oxidized. Complete studies have not been made of the limiting Eh for growth, although the results with L-cysteine (see below) indicate that none of the fungi except _Armillarie mellea_ (see below) grow well at an Eh more negative than about + 400 millivolts.

# TABLE 14

## INITIAL OXIDATION-REDUCTION POTENTIALS OF UNINOCULATED NUTRIENT MEDIA

Media and/or nitrogen components of media are those used in Tables 10 and 11. Nitrogenous components added to basal medium (Table 1) to 0.012% total nitrogen, except ammonium nitrate to 0.012% ammonium nitrogen, and DL- amino acids to 0.024% total nitrogen. Media autoclaved, shaken for one hour, and allowed to stand one-half hour prior to Eh determinations. Eh readings based on hydrogen electrode.

| Medium or Component | Average pH | Eh Average + millivolts | Eh Range + millivolts |
|---|---|---|---|
| Malt extract (1%) | 4.85 | 431 | 426 - 436 |
| Casein hydrolysate | 5.45 | 466 | 440 - 476 |
| Urea | 5.40 | 436 | 431 - 438 |
| $(NH_4)_2CO_3$ | 5.50 | 416 | 350 - 421 |
| $(NH_4)_2SO_4$ | 5.50 | 446 | 436 - 461 |
| $NH_4NO_3$ | 5.48 | 456 | 433 - 466 |
| $NH_4Cl$ | 5.42 | 410 | 406 - 425 |
| Glutamic acid | 5.52 | 417 | 413 - 421 |
| Glycine | 5.29 | 506 | 496 - 517 |
| B- alanine | 5.38 | 456 | 446 - 471 |
| L- leucine | 5.22 | 466 | 447 - 476 |
| L- cysteine | 5.45 | 356 | 345 - 368 |
| L- cystine | 5.25 | 396 | 394 - 406 |
| L- tyrosine | 5.30 | 441 | 436 - 456 |
| L- tryptophane | 5.35 | 456 | 451 - 461 |
| L- proline | 5.30 | 476 | 470 - 484 |
| L- histidine | 5.29 | 433 | 426 - 438 |
| L- asparagine | 5.45 | 486 | 466 - 506 |
| L- lysine | 5.29 | 440 | 431 - 451 |
| L- arginine | 5.40 | 491 | 484 - 498 |
| DL- valine | 5.42 | 501 | 483 - 504 |
| DL- isoleucine | 5.28 | 464 | 457 - 470 |
| DL- serine | 5.31 | 477 | 469 - 486 |
| DL- threonine | 5.30 | 476 | 467 - 483 |
| DL- methionine | 5.30 | 428 | 422 - 440 |
| DL- aspartic acid | 5.45 | 496 | 491 - 507 |
| DL- phenylalanine | 5.42 | 485 | 471 - 477 |
| DL- norleucine | 5.28 | 486 | 476 - 501 |
| DL- ornithine | 5.18 | 507 | 504 - 515 |

A series of studies of the Eh changes in shake culture during the growth cycle of one to three weeks, depending upon the organism, have yielded little consistent or significant data. Presumably because of being continuously aerated, shake cultures did not show the striking drop in Eh which is characteristic of microbial growth in stationary cultures. In general, daily Eh readings were rather variable as regards replicate samples, the same medium or the same organism, and showed little consistency relative to the Eh trend in growing cultures. Much, perhaps most, of this variation was a reflection of the inconstancy of the determination itself, in cultures in which there was a continual interchange of gases with the atmosphere. It appears that except in special cases, routine Eh determinations in culture are not significant.

Table 11 shows that none of the fungi used grew in the basal medium plus L-cysteine through very many serial subcultures. Studies of the Eh of this medium yielded the explanation for lack of growth. This amino acid is commonly used in bacteriological media to obtain reduced conditions for the growth of anaerobes, and in our work produced an Eh too low ( + 350 m.v.) even in aerated culture, for growth of the aerobic wood rots. All other amino acid media were more highly oxidised, with an Eh of + 500 m.v. (Table 14). Good growth of ten organisms tested was obtained when ascorbic acid, an oxidising agent which is not utilized but which increases the Eh, was added to the cysteine medium to oxidise it to an Eh of + 450 m.v. Growth also could be obtained by decreasing the concentration of cysteine, resulting in a less reduced medium. It is apparent, then, that L-cysteine is a nutritionally - utilisable form of nitrogen under an environmental condition comparable with that for the other amino acids, i.e., in a highly oxidised medium.

An exception to the above statement that the organisms require a highly oxidized medium for growth is *Armillaria mellea*, which was studied separately, because it originally apparently did not utilise amino acids (see Table 10) and therefore was not studied in Table 11. Good growth of this organism was obtained only in a less highly oxidised medium. For example, the basal medium plus cysteine in a concentration of 0.024% total nitrogen, Eh about + 300 m.v., supported growth comparable in amount to that of other wood rots in highly oxidised media. Thus, what appear to be different *nutritional* requirements of certain organisms may in some cases be a reflection of special *environmental* requirements.

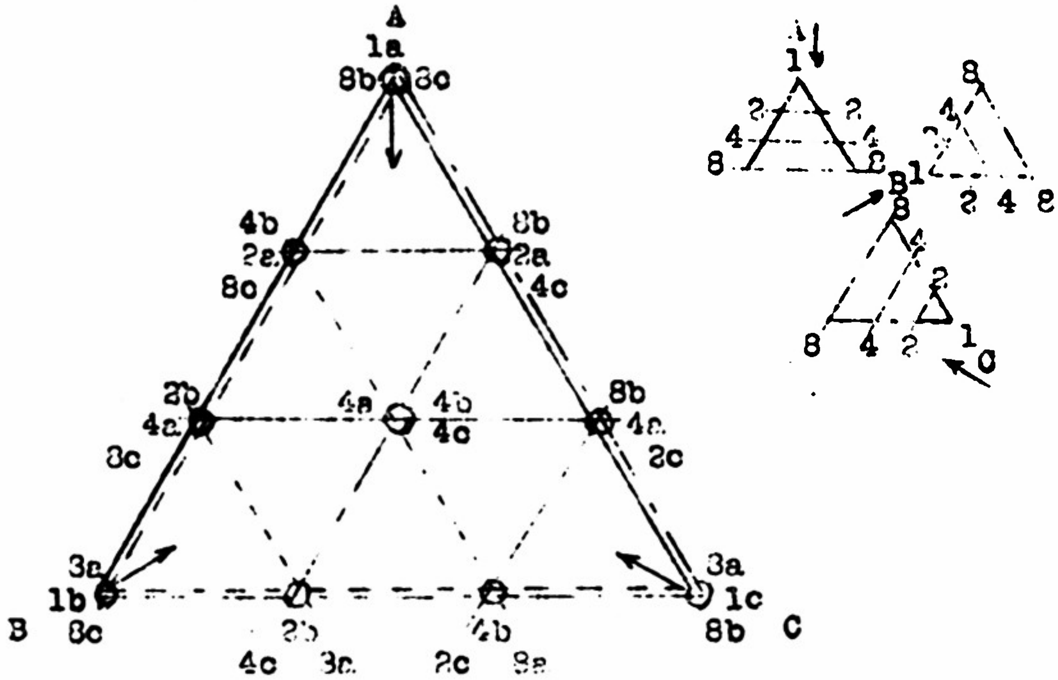
## DEVELOPMENT OF SYNTHETIC MEDIA " OPTIMAL" FOR GROWTH

The original basal synthetic medium (Table 1) supports good growth of most of our fungi (exceptions: A. mellea, P. vaillantii, P. luteofibrata, F. officinalis (Table 10)), but probably is not qualitatively or quantitatively optimal for any species. It should be regarded as containing the minimum essential nutrients in useable concentrations. It seemed desirable to develop, for some of the organisms, synthetic media which were at least quantitatively optimal in the minimum essential nutrients, rather than constitutents which enhance growth, but are not essential.

The "triangle" (3-variable) method (Hildebrandt etal, 1946) was used as a basis for determining the optimal concentration of each nutrient in the presence of the others, for the constituents of the original basal medium plus glutamic acid as a source of nitrogen. Modifications of the triangle method -- the 6-variable and 12-variable systems -- also were developed and used. All work was carried out in shake flasks and with the other techniques given under Methods.

In the triangle method (Fig. 5), concentrations of three nutrients are varied simultaneously (keeping the concentrations of other constituents constant), usually doubling the concentrations stepwise within the ranges covered. For the first approximation to the optimal concentration of each of the three variables (e.g., glucose, glutamic acid, thiamin) a wide range of concentrations was used, and the optimum concentration was given by the flask showing the greatest amount of growth (dry weight of mycelium). Closer and closer approximations to the "true" optimum for the three nutrients was then obtained by using narrower ranges about the optima first determined. After the optima for the first three nutrients were established with the desired accuracy, the optimal concentrations of

**The "triangle" method for approximating the optimal concentrations of three variables in nutrition studies.**




Given three nutrients, A, B and C: it is desired to determine the optimal concentration of each in the presence of the others. Ten flasks are set up as shown (O) in the large triangle, each flask containing the indicated relative concentrations (1a, 4b, 2c, etc.) of substances A, B and C. For the first approximation of the optimum, each substance is used in 1, 2, 4 and 8 times some chosen concentration of A, B and C, as shown in the small triangles. The small triangles are, in effect, superimposed to form the large triangle. The absolute values of 1a, 1b and 1c need not necessarily be the same. The first approximation to the optimal concentrations of the three nutrients is given by the flask (combination of concentrations) which produces the greatest amount of growth. Closer and closer approximations may then be obtained by using other or narrower ranges of concentrations about the optima previously determined. A greater or lesser number of concentrations (flasks) may be used in a set than is illustrated above.

FIGURE 5

Reference: Hildebrandt, Riker and Duggar, Am. J. Botany, 33, 591-97, 1946.

another set of three nutrients were determined in the same manner, keeping constant the optima first determined. Theoretically, at least, the procedure may be repeated, using all possible combinations and concentrations of nutrients, until an absolute optimal medium for an organism is reached. With this triangle method we have used either 5 or 7 concentrations of each of three variables, which required 15 or 28 flasks, respectively, per run.

In the triangle method the variables are run only in groups of three, so that interaction of each nutrient of the complete medium with all of the others cannot be studied at the same time. The 12-variable system was developed so that concentrations of all 12 constituents (including pH) of the basal medium could be varied and studied simultaneously. Six variables (glucose, glutamic acid, thiamin, potassium phosphate, magnesium sulfate and pH) were used in 13 concentrations, and the other six (zinc, iron, manganese, molybdenum, copper and boron) in 9 concentrations. This required 43 flasks per run, and more and better data was obtained in less time than with the triangle method.

The 6-variable system is similar to the 12-variable method, except that the trace elements are not included as variables. Quantitative optima were established for the trace elements by the 12-variable system. It was found, however, that the other six constituents had a relatively much greater effect on growth, so that for all practical purposes the concentrations of trace elements in the original basal medium were "optimum". Hence much work could be saved in developing "optimal" media by studying only the six other constituents. Using 9 concentrations of each of these six variables, 37 flasks per run were required.

We have developed more nearly optimal media for 9 organisms, using the same concentrations of trace elements as in the original basal medium, but varying the concentrations of the other constituents. The results are shown in Table 15, based on the 6-variable method. Very similar data have been obtained with the triangle and the 12-variable methods.

It is apparent that even in these "optimal" media which contain only essential nutrients, there are very large increases in the amount of growth compared with the "standard" (original) medium. This varies from 3-fold with *D. quercina* to 37-fold with *A. mellea*. From the data obtained by the various methods, it has been found in general that the factors most critical in increasing growth are the concentrations of glucose, nitrogen and potassium phosphate, and to some extent the proper ratio of potassium phosphate to magnesium sulfate.

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TABLE 15

"OPTIMAL" MEDIA FOR GROWTH OF CERTAIN WOOD-ROTTING FUNGI IN SHAKE CULTURE

Criterion of "growth" is dry weight of mycelium produced per 70 ml. of medium in 250-ml. Erlenmeyer flask. "Initial pH" is that of medium before inoculation and is optimum for each organism. Temperature of incubation = 28°C. Six variables (all except trace elements) run at same time.

| | A | B | | | | | |
|---|---|---|---|---|---|---|---|
| | | Composition of media optimal for indicated brown rots | | | | | |
| Constituent | Original basal medium (Table 1) | P. palustris | L. trabea | | D. quercina | T. serialis | F. subroseus |
| Glutamic acid * | 0.012 | 0.1 | 0.02 | | 0.04 | 0.12 | 0.08 |
| Glucose * | 1.00 | 12.0 | 8.0 | | 10.0 | 16.0 | 10.0 |
| $KH_2PO_4$ * | 0.15 | 0.5 | 0.4 | | 0.4 | 0.5 | 0.6 |
| $MgSO_4$ * | 0.05 | 0.05 | 0.1 | | 0.1 | 0.05 | 0.04 |
| Thiamine ** | 1.00 | 2.0 | 4.0 | | 3.5 | 2.5 | 2.5 |
| Zn ** | 0.07 | Same as in original basal medium (A) | | | | | |
| Fe ** | 0.05 | | | | | | |
| Mo ** | 0.01 | | | | | | |
| B ** | 0.10 | | | | | | |
| Mn ** | 0.01 | | | | | | |
| Cu ** | 0.01 | | | | | | |
| Initial pH | 5.5 | 5.2 | 4.4 | | 4.8 | 4.8 | 4.2 |
| Culture age, days | 7, 14 | 14 | 7, | 14 | 14 | 7 | 14 |
| Mg. growth in A | | 45 | 78 | 92 | 152 | 69 | 74 |
| Mg. growth in B | | 402 | 652 | 735 | 482 | 789 | 298 |
| Growth, B/A x 100 | | 894% | 836% | 800% | 317% | 1145% | 403% |

* Expressed in %. For glutamic acid, as % nitrogen.

** Expressed as mg. per liter of medium.

TABLE 15(CONCLUDED)

| Constituent | A<br>Original basal medium (Table 1) | B<br>Composition of media optimal for indicated white rots<br>F. geotropus | P. tulipiferus | A. mellea | L. tigrinus |
|---|---|---|---|---|---|
| Glutamic acid * | 0.012 | 0.12 | 0.14 | 0.06 | 0.10 |
| Glucose * | 1.00 | 4.0 | 20.0 | 14.0 | 12.0 |
| $KH_2PO_4$ * | 0.15 | 0.8 | 0.4 | 0.3 | 0.6 |
| $MgSO_4$ * | 0.05 | 0.025 | 0.1 | 0.2 | 0.04 |
| Thiamine ** | 1.00 | 1.5 | 1.0 | 3.0 | 2.0 |
| Zn ** | 0.07 | Same as in original basal medium (A) | | | |
| Fe ** | 0.05 | | | | |
| Mo ** | 0.01 | | | | |
| B ** | 0.10 | | | | |
| Mn ** | 0.01 | | | | |
| Cu ** | 0.01 | | | | |
| Initial pH | 5.5 | 5.2 | 6.0 | 5.0 | 5.4 |
| Culture age, days | 7, 14, 21 | 7 | 7, 14 | 21 | 7 |
| Mg. growth in A | | 125 | 66, 90 | 25 | 90 |
| Mg. growth in B | | 1350 | 259, 552 | 925 | 924 |
| Growth, B/A x 100 | | 1080% | 392%, 613% | 3700% | 1025% |

* Expressed in %. For glutamic acid, as % nitrogen.

** Expressed as mg. per liter of medium.

## SPECIAL GROWTH CONDITIONS OF CERTAIN WOOD ROTS

It will be noted from Table 10 that three organisms — *P. officinalis*, *P. vaillantii* and *P. luteofibrata* — could not grow in any medium (nitrogen source) except malt extract. In addition, *A. mellea* did not originally grow well even in malt extract, and did not originally grow at all in the glutamic acid medium or other nitrogen sources except casein hydrolysate. The special growth requirements of these form organisms were therefore investigated.

The poor growth of *A. mellea* apparently was due to a combination of factors. First, continued subculture so "trained" the organism that fair growth was finally obtained in malt extract and in glutamic acid medium (Table 10). The mechanism of this training was not investigated. Secondly, poor growth resulted in part from the too high degree of oxidation of most media (Eh = +400 to +500 m.v., Table 14). Good growth was obtained in the synthetic medium with cysteine (Eh = +300 m.v.), which is a reducing agent, as mentioned above in the section on oxidation - reduction potentials.

Growth of *P. luteofibrata* was limited in malt extract (Table 10) and the organism died out upon subculture in the other media. Lack of growth apparently is due largely, at least, to acid production. Daily pH readings showed that the pH drops very rapidly, acid being produced as the result of only a few milligrams of growth. By readjusting the pH at 2-day intervals to the original pH 5.5, good growth was obtained in a variety of media. A buffer solution sufficiently concentrated to maintain the original pH inhibited growth of the organism.

As regards the poor growth of *P. vaillantii* and *P. officinalis*, certain essential nutrients were lacking in the original basal medium. This was determined by developing special synthetic media based on the "standard" medium. These media are shown in Table 16, where it is seen that both organisms require adenine in the basal medium, and that *P. vaillantii* has additional minimum requirements for biotin and riboflavin in addition to thiamin.

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TABLE 16

SYNTHETIC MEDIA FOR WOOD-ROTTING FUNGI NOT GROWING IN "STANDARD" SYNTHETIC MEDIUM.

| Constituent | A<br>Original basal medium (Table 1) | B<br>Composition of media for growth of indicated fungi. All cultures 14 days old.<br>F. officinalis | <br><br>P. vaillantii |
|---|---|---|---|
| Glutamic acid * | 0.012 | 0.10 | 0.06 |
| Glucose * | 1.00 | 10.0 | 10.0 |
| $KH_2PO_4$ * | 0.15 | 0.7 | 0.5 |
| $MgSO_4$ * | 0.05 | 0.03 | 0.05 |
| Thiamine ** | 1.00 | 2.0 | 3.0 |
| Zn ** | 0.07 | | |
| Fe ** | 0.05 | | |
| Mo ** | 0.01 | Same as in basal original medium (A) | |
| B ** | 0.10 | | |
| Mn ** | 0.01 | | |
| Cu ** | 0.01 | | |
| Adenine ** | 0 | 18.0 | 20.0 |
| Additional requirements | 0 | 0 | Biotin — 2 gammas per liter; riboflavin — 2 mg. per liter |
| Initial pH | 5.5 | 4.5 | 5.0 |
| Mg. growth in malt extract | | 63 | 96 |
| Mg. growth in B | | 296 | 115 |
| Growth, B/malt ext. x 100 | | 470% | 120% |

* Expressed in %. For glutamic acid, as % nitrogen.

** Expressed as mg. per liter of medium.

## VITAMIN RELATIONSHIPS IN SYNTHETIC MEDIA

It has already been found that: 1. T. serialis and P. rubescens require no vitamins in synthetic media (Tables 10 and 11). 2. All other species studied require thiamin (Tables 10 and 11), although P. palustris and P. tulipiferus, at least, can substitute biotin for thiamin (Table 4). 3. Vitamins and nutrilites other than thiamin may stimulate growth, but generally are not essential (Table 4). 4. F. officinalis and P. vaillantii require adenine in addition to thiamin, and the latter also needs biotin and riboflavin. Further studies have been made of some of these relationships of vitamins to growth.

Substitution of biotin for thiamin. Following up a previous observation that with two organisms biotin could be substituted for thiamin, we have studied the rest of our wood-rotting fungi in this regard in the basal medium with glutamic acid but without thiamin. Preliminary studies showed that 2 gammas per liter of biotin gave approximatly maximum growth, so this concentration was used throughout. The results are tabulated in Table 17. For most organisms, biotin gave continued growth in serial subculture, although the amount of growth at the end of 7 days' incubation was not usually as great as with thiamin. Certain organisms died out in biotin, namely, L. saepiaria, P. betulinus, P. immitus, P. cocos and P. incrassata, P. luteofibrata, A. mellea, F. officinalis and P. vaillantii which have special nutrient requirements (see previous section) did not grow at all with biotin. These results suggest that most of the organisms have two metabolic pathways for their basic metabolic activities. Microbiological essays for thiamin were carried out on all culture filtrates before and after growth, but no

## TABLE 17

### EFFECT OF SUBSTITUTION OF BIOTIN FOR THIAMINE ON GROWTH OF WOOD ROTS IN SHAKE CULTURE AT 28°C.

Basal medium (Table 1) plus glutamic acid (to nitrogen of 0.012%), with thiamine (1 mg. per liter) and with biotin (2 gamma per liter) substituted for thiamine. Medium was adjusted to approximately optimum pH for each organism (see Table 7). Standard inoculum used. Growth response expressed as milligrams of dry weight of mycelium per 70 ml. of medium. All data are averages of duplicate, 7-day cultures. Figures for growth in thiamine are third-transfer data; for growth in biotin, Column A is third-transfer and Column B is ninth-transfer, unless otherwise indicated. A superscript after a zero shows the number of the serial transfer in which growth died out.

| Organism | Mycelial weight - milligrams with thiamine | with biotin A | with biotin B |
|---|---|---|---|
| Brown rots - | | | |
| Coniophora cerebella | 24 | 20 | 17 |
| Daedalea quercina | 19 | 18 | 20 |
| Fomes officinalis | 0 | 0 | 0 |
| Fomes meliae | 95 | 65 | 62 |
| Fomes roseus | 32 | 19 | 22 |
| Fomes subroseus | 77 | 50 | 54 |
| Lentinus lepideus | 20 | 12 | 14 |
| Hydnum pulcherrimum | 24 | 22 | 21 |
| Lenzites saepiaria | 18 | 5 | $0^4$ |
| Lenzites striata | 10 | 26 | 24 |
| Lenzites trabea | 89 | 33 | 32 |
| Merulius lacrymans | 33 | 20 | 19 |
| Polyporus betulinus | 86 | 5 | $0^4$ |
| Polyporus immitus | 24 | 3 | $0^4$ |
| Polyporus palustris | 86 | 45 | 51 |
| Polyporus spraguei | 28 | 29 | 30 |
| Polyporus sulphureus | 36 | 29 | 28 |
| Polyporus schweinitzii | 35 | 22 | 22 |
| Poria oleraceae | 110 | 77 | 75 |
| Poria cocos | 30 | 9 | $0^4$ |
| Poria incrassata | 45 | $0^2$ | 0 |

(continued on next page)

TABLE 17 (CONCLUDED)

| Organism | Mycelial weight - milligrams with thiamine | with biotin A | B |
|---|---|---|---|
| Brown rots (cont'd) | | | |
| Poria luteofibrata | 24 | 0[2] | 0 |
| Poria monticola | 98 | 62 | 60 |
| Poria nigra | 115 | 30 | 33 |
| Poria vaillantii | 0 | 0 | 0 |
| Poria xantha | 70 | 59 | 58 |
| Ptychogaster rubescens | 128 | 67 | 69 |
| Trametes serialis | 40 | 28 | 30 |
| Trámetes malicola | 43 | 22 | 24 |
| White rots - | | | |
| Armillaria mellea | 0 | 0 | 0 |
| Fomes annosus | 66 | 39 | 38 |
| Fomes fomentarius | 4 | 5 | 6 |
| Fomes geotropus | 58 | 30 | 26 |
| Fomes pini | 38 | 18 | 17 |
| Lentinus tigrinus | 100 | 16 | 18 |
| Peniophora gigantea | 115 | 40 | 38 |
| Polyporus abietinus | 134 | 22 | 27 |
| Polyporus fumosus | 20 | 25 | 23 |
| Polyporus versicolor | 92 | 84 | 87 |
| Poria subacida | 60 | 28 | 29 |
| Polyporus anceps | 54 | 27 | 29 |
| Polyporus tulipiferus | 76 | 34 | 31 |

1-2

thiamin was found. Thiamin assays of the mycelium of the organisms after growth were somewhat inconclusive, as good digests of the mycelium could not be made. The assay data indicate that a few organisms contain traces of thiamin in the mycelium, but apparently appreciable quantities of thiamin are not synthesized during growth with biotin.

Effect of components of the thiamine molecule. Previous preliminary data had suggested that certain wood rots could utilize one or the other component of the thiamine molecule, instead of requiring the whole molecule. Tests with 15 selected organisms were run to investigate this point, using the basal medium plus glutamic acid. The following were added separately to this medium: thiamine (control), thiazole, "pyrimidine" (2-methyl-5-bromomethyl-4-aminopyrimidine dihydrobromide instead of the chloride), and thiazole plus pyrimidine. The thiazole and pryimidine were used in concentrations equimolar to thiamine. The results are tabulated in Table 18.

It is apparent that the organisms fall into five groups as regards their ability to utilize thiamine components. Group 1 organisms require the whole thiamine molecule; Group 2 organisms can grow in thiazole plus pyrimidine, but not in either of the separate components; species in Group 3 utilize thiazole plus pyrimidine, and thiazole alone, but not pyrimidine alone; Group 4 species grow in thiazole plus pyrimidine, and in pyrimidine alone, but not in thiazole alone; Group 5 contains species which can utilize thiazole plus pyrimidine, thiazole alone, or pyrimidine alone, in addition to the thiamine molecule. These groups are reflections of various synthetic mechanisms in the different species of brown and white rots.

TABLE 18

EFFECT OF COMPONENTS OF THE THIAMINE MOLECULE ON GROWTH OF WOOD ROTS IN SHAKE CULTURE AT 28°C.

Basal medium (Table 1) plus glutamic acid (to nitrogen of 0.012%), with thiamine control (1 mg. per liter) and with thiazole (0.42 mg. per liter) and pyrimidine (as bromide instead of chloride, 0.84 mg. per liter) substituted for thiamine in concentrations equimolar to thiamine. Media were adjusted to approximately optimum pH for each organism (see Table 7). Standard inoculum used. Growth response expressed as milligrams of dry weight of mycelium per 70 ml. of medium. No growth without added nutrilites. All data are averages of duplicate, 7-day cultures, after three serial transfers in the given medium. Brown rots and white rots indicated by (B) and (W) respectively.

| | Mat weight - milligrams | | | |
|---|---|---|---|---|
| Organism | Thiamine | Thiazole + Pyrimidine | Thiazole | Pyrimidine |
| **Group 1** | | | | |
| Lentinus lepideus (B) | 22 | 0 | 0 | 0 |
| Polyporus immitus (B) | 18 | 0 | 0 | 0 |
| **Group 2** | | | | |
| Fomes meliae (B) | 100 | 40 | 0 | 0 |
| Lenzites trabea (B) | 90 | 46 | 0 | 0 |
| **Group 3** | | | | |
| Polyporus betulinus (B) | 93 | 89 | 80 | [illegible] |
| Polyporus tulipiferus (W) | 85 | 83 | 130 | 0 |
| **Group 4** | | | | |
| Trametes malicola (B) | 38 | 25 | 0 | 20 |
| Trametes serialis (B) | 72 | 70 | 0 | 35 |
| **Group 5** | | | | |
| Fomes roseus (B) | 35 | 31 | 23 | 25 |
| Polyporus anceps (W) | 62 | 43 | 36 | 35 |
| Polyporus schweinitzii (B) | 38 | 23 | 20 | 22 |
| Fomes annosus (W) | 68 | 70 | 74 | 68 |
| Fomes pini (W) | 26 | 25 | 30 | 25 |
| Fomes subroseus (B) | 75 | 73 | 70 | 65 |
| Polyporus palustris (B) | 92 | 88 | 84 | 86 |

Effects on growth of vitamin $B_{12}$ and of p-aminobenzoic acid. Neither of those vitamins had previously been tried, and vitamin $B_{12}$ ("Cobione", Merck) is only newly available. They were used in the synthetic medium (Table 1) with glutamic acid (0.012% nitrogen) and thiamine (1 mg. per liter). Vitamin $B_{12}$ was used in a concentration of 0.1 gamma per liter, and p-a-b as 2 mg. per liter.

The following organisms, tested with vitamin $B_{12}$ plus thiamine, showed no difference in growth in the presence of this vitamin than in its absence (thiamine only): P. tulipiferus, P. incrassata, P. monticola, P. palustris, L. lepideus, F. subroseus, L. trabea, P. anceps, F. roseus, F. meliae, F. officinalis, P. xantha, P. cocos, P. nigra, P. spraguei, and P. vaillantii. (The latter two organisms did not grow with thiamin.)

The following organisms, tested with p-aminobenzoic acid plus thiamine, showed no difference in growth in the presence of this nutrilite than in its absence (thiamine only): P. tulipiferus, P. anceps, P. palustris, L. lepideus.

## UTILIZATION OF DIFFERENT CARBON COMPOUNDS

It has previously been shown (section on Utilisation of different forms of organic and inorganic nitrogen in synthetic media) that P. palustris and P. tulipiferus could not use any amino acid as its sole source of carbon, and that none of our wood rots could use l-glutamic acid as a sole source of carbon. Carbon sources other than amino acids and glucose have been studied as regards their utilisation by P. palustris and P. tulipiferus, adding the compounds to the basal medium (plus glutamic acid) in place of glucose.

The concentration of each carbon source was such as to give a carbon concentration equivalent to that in the standard 1% glucose. Utilisation of each compound is expressed as milligrams (dry weight) of mycelium in the third serial subculture in standard shake flasks. The results are shown in Table 19. It is apparent that both organisms utilise the same carbon compounds, and that all compounds are utilised except certain organic acids - acetic, citric, maleic, and tartaric. Pyruvic and succinic acids apparently were metabolised, although to a lesser degree than most of the other carbon compounds. The amount of growth in glycerol is striking.

TABLE 19

Growth of *P. palustris* and *P. tulipiferus* in shake culture at 28° C. in the basal medium with various carbon compounds substituted for glucose.

Growth expressed as milligrams (dry weight) of mycelium per 70 ml. of medium. Data are averages of duplicate, 7-day cultures, after three serial transfers in the given nutrient. Original inoculum from basal medium with glucose.

| Carbon source | *P. palustris* mg. | *P. tulipiferus* mg. |
|---|---|---|
| Acetic acid | 0 | 0 |
| Citric acid | 0 | 0 |
| Fructose | 63 | 14 |
| Galactose | 26 | 39 |
| Glycerol | 149 | 138 |
| Lactic acid | 37 | 29 |
| Lactose | 41 | 17 |
| Maleic acid | 0 | 0 |
| Maltose | 63 | 84 |
| Pyruvic acid | 19 | 17 |
| Succinic acid | 33 | 29 |
| Sucrose | 43 | 17 |
| Starch, soluble | 63 | 69 |
| Tartaric acid | 0 | 0 |
| Glucose (control) | 96 | 77 |

Studies of carbohydrate utilization (oxidation) also were made using the Warburg respirometer. Utilization is measured in terms of oxygen uptake under standard conditions (exogenous respiration). Only *P. palustris* has been studied in this regard, as great difficulty was experienced in developing a proper technique with the wood rots. The manometric technique is a more delicate one than fermentation for studying carbohydrate utilization.

For consistent results in measuring the oxygen uptake, the inoculum must be properly prepared and standardized. After many trials to obtain a uniform mycelial suspension with a high exogenous respiration, the following procedure was the best that could be devised. From a stock culture of the organism on a potato dextrose agar slant, transfer a small piece of mycelium to 70 ml. of 2 % malt extract in a 250-ml. Erlenmeyer flask. Incubate at 28° C. on the shaking machine for 5 days to produce pellets. Transfer the contents of the flask to a Waring blendor and blend the pellets for one minute. Transfer an aliquot of the blended suspension to a 15 ml. centrifuge tube and spin down for two minutes at 2000 r.p.m. Discard the supernatant, resuspend the cells in distilled water, and centrifuge again. Resuspend the cells in distilled water to give a suspension of about 10% by volume. One ml. of this suspension is then used to inoculate 70 ml. of 2% malt extract in a 250-ml. Erlenmeyer flask for starting the final inoculum. Incubate on the shaking machine for 48 hours to obtain the basic inoculum for the Warburg determinations. The pellets may now either be blended in a Waring blender for 5 seconds, or transferred intact to a 50 ml. centrifuge tube. Unblended pellets give somewhat better results later. In either case centrifuge at 2000 r.p.m. for 2 minutes, wash with distilled water, and repeat three times. After the final centrifugation resuspend the cells in 50 ml. of phosphate buffer, pH 5.5, transfer to a 250-ml. Erlenmeyer flask, and put on the shaking machine for 24 hours to starve the cells in order to decrease endogenous respiration. Centrifuge the cells and resuspend in phosphate buffer to give a suspension of approximately 20% by volume. One ml. of this inoculum is used per flask in the Warburg apparatus.

Using the above standard inoculum, the oxygen uptake of *Polyporus palustris* was determined manometrically with several carbohydrates in 1% concentration as substrates. The *endogenous* respiration of the starved cells was measured at the same time, and the *exogenous* figures corrected for the endogenous value. Table 20 shows the corrected exogenous respiration values, expressed as micro-liters of oxygen per milligram of cells per hour. The values shown are averages of triplicate determinations. (*Endogenous* values varied between about 1 and 2 micro-liters $O_2$/mg./hr.)

TABLE 20

Exogenous respiration of P. palustris on various carbohydrates, corrected for endogenous respiration. Oxygen uptake expressed as microliters $O_2$/mg./hr. Figures are averages of triplicate determinations.

| Substrate | Average µl./mg./hr. | Standard Deviation |
| --- | --- | --- |
| Fructose | 2.29 | 0.54 |
| Galactose | 2.14 | 0.12 |
| Sucrose | 2.13 | 0.06 |
| Glucose | 2.02 | 0.12 |
| Maltose | 1.94 | 0.16 |
| d-Mannose | 1.92 | 0.11 |
| Dextrin | 1.73 | 0.12 |
| d-Xylose | 1.67 | 0.12 |
| Glycogen | 1.60 | 0.14 |
| Arabinose | 1.49 | 0.17 |
| Cellobiose | 1.20 | 0.16 |
| Lactose | 0.49 | 0.05 |
| d-Ribose | 0.43 | 0.07 |
| l-Rhamnose | 0.16 | 0.05 |

The data in Table 20 show that this organism utilizes all of the carbohydrates, although lactose, ribose and rhamnose are used rather poorly. (All oxygen uptake values for this organism are rather low compared with most bacteria, other fungi and other types of cells. Whether this is normal, or whether the best technique has not been developed, is not known.)

MR 034150

## STUDIES OF GROWTH IN AERATED BOTTLE CULTURES

These studies were carried out to compare certain aspects of growth in large aerated cultures with that in shake culture, to determine the amount of growth and acid produced when the pH was and was not adjusted periodically, and to determine the amount of acid produced (titratable acidity) during growth and fermentation. The nitrogen sources - - glutamic acid, ammonium sulfate, ammonium nitrate and ammonium carbonate - - were added singly to the basal medium (Table 1) in a concentration to give 0.012% total nitrogen in each case. The concentration of glucose used was only 0.5% instead of 1% as used in shake culture. Thiamine was added as 1 mg. per liter, and adenine as 13 mg. per liter, the latter to stimulate growth and fermentation. One-liter quantities of medium were used in 2-quart bottles (Fig. 2, above), and incubation was at 28° for 7 days. Sterile air was forced through the cultures at a rate of approximately 2 liters per minute.

The following organisms were studied in aerated bottle culture: P. palustris, L. lepideus, P. rubescens, P. tulipiferus, and F. annosus. Two series of tests were run concurrently - in one set the pH was adjusted back to the original 5.5 daily, while in the other set no adjustment was made. Determinations of pH, titratable acidity and mycelial weights (dry basis) were made at the end of 7 days. Table 21 shows the results of these experiments.

The data in Table 21 show that there was, in general, less difference than might be expected in the amount of growth whether the pH was adjusted daily or not adjusted at all during incubation. These differences were most marked in ammonium sulfate, in which all organisms produced appreciably more total growth in 7 days when the pH was adjusted daily. P. rubescens in glutamic acid also is noteworthy. There were greater differences in the amount of acid produced as regards pH adjustment, although these differences were not consistent even with a given organism.

NR 034159

TABLE 21

GROWTH OF WOOD ROTS IN AERATED BOTTLE CULTURE

Culture medium: basal medium with glucose (0.5%), thiamine, adenine, nitrogen source (0.012% total nitrogen). Original volume of medium: 1 liter; original pH = 5.5. Aeration rate: 2 liters per minute. NaOH given as total ml. of N/0.25 used to neutralize 10 ml. of medium during 7 days.

| Nitrogen source and organism | pH adjusted daily: Mycelial weight - 7 days | pH adjusted daily: Final pH - 7 days | pH adjusted daily: NaOH to neutralize - 7 days | pH not adjusted: Mycelial weight - 7 days | pH not adjusted: Final pH - 7 days | pH not adjusted: NaOH to neutralize - 7 days |
|---|---|---|---|---|---|---|
| | gms. | pH | ml. | gms. | pH | ml. |
| **Glutamic acid** | | | | | | |
| P. palustris | 0.6008 | 2.98 | 1.47 | 0.8688 | 2.05 | 1.1 |
| L. lepideus | 0.5917 | 4.92 | 0.05 | 0.6513 | 4.88 | 0.05 |
| P. rubescens | 1.8702 | 6.00 | - | 0.8821 | 6.05 | - |
| P. tulipiferus | 3.0000 | 5.90 | 0.26 | 3.3500 | 5.50 | 0 |
| F. annosus | 0.2820 | 6.50 | -0.06 | 0.3990 | 8.20 | - |
| **$(NH_4)_2SO_4$** | | | | | | |
| P. palustris | 0.4533 | 2.60 | 0.51 | 0.3581 | 2.62 | 1.13 |
| L. lepideus | 0.6979 | 3.32 | 0.16 | 0.2806 | 3.72 | 0.09 |
| P. rubescens | 1.5382 | 5.10 | 0.20 | 1.0420 | 3.02 | 0.80 |
| P. tulipiferus | 1.1520 | 6.00 | -0.05 | 0.9450 | 5.50 | -0.10 |
| F. annosus | 0.1930 | 5.70 | -0.02 | 0.1030 | 5.50 | 0 |
| **$NH_4NO_3$** | | | | | | |
| P. palustris | 0.6102 | 2.22 | 0.27 | 0.4541 | 2.12 | 0.11 |
| L. lepideus | 0.4063 | 6.60 | - | 0.4198 | 7.20 | - |
| P. rubescens | 0.8526 | 6.50 | - | 0.7487 | 6.10 | - |
| P. tulipiferus | 0.1040 | 5.95 | 0.14 | 0.3020 | 5.50 | 0 |
| F. annosus | 0.0380 | 6.20 | -0.06 | 0.0090 | 5.00 | 0.01 |
| **$(NH_4)_2CO_3$** | | | | | | |
| P. palustris | - | - | - | 1.092 | 2.90 | 1.2 |
| L. lepideus | - | - | - | - | - | - |
| P. rubescens | 0.0316 | 6.50 | 0.42 | 0.8317 | 3.50 | 0.37 |
| P. tulipiferus | 0.1270 | 4.90 | 0.14 | 0.1010 | 4.90 | 0.11 |
| F. annosus | 0.0310 | 6.20 | -0.10 | 0.0150 | 6.50 | -1.10 |

In one medium an organism, e.g., P. palustris, produced more total acid when the pH was adjusted, while in another medium the same fungus produced more acid when the pH was not adjusted from day to day. It appears impossible to generalise as regards the effect of adjusting pH during growth.

The results show that in glutamic acid, P. tulipiferus made by far the best growth, and the amount was twice as great as any other organism in any nitrogen source. In ammonium sulfate, P. tulipiferus and P. rubescens were nearly the same in production of mycelium, while in ammonium nitrate, P. rubescens produced nearly twice as much growth as any other organism in that compound. The greatest amount of growth in ammonium carbonate was produced by P. palustris. F. annosus grew best in glutamic acid, while L. lepideus also favored glutamic acid over the inorganic nitrogen compounds. Thus the various fungi differ among themselves as regards their best nitrogen source, although in general glutamic acid supported more growth than the inorganic nitrogen compounds.

Whereas in shake culture acid is invariably produced (Tables 8 and 9 above), in the aerated cultures the pH was more alkaline at the end of 7 days with certain organisms in various nitrogen sources. P. palustris was consistently the best acid-producer in all media, while the other organisms sometimes produced an acid reaction and sometimes an alkaline one by the end of the incubation period. A possible explanation for the alkaline reaction may be that because of the relatively high rate of aeration in the bottle cultures, organic acids which are produced may be further oxidized, forming alkaline carbonates.

The above studies show that controlled growth in mass culture is feasible. Pellet size, and to some extent the total amount of growth, is controlled by the rate of aeration. The compressed air is readily sterilized by passage through dry, sterile fiber-glass filters. The method is useful in the quantity production of mold enzymes and fermentation products for analysis.

## CHEMICAL PRODUCTS OF FERMENTATION AND/OR SYNTHESIS OF THE WOOD ROTS

In addition to cellulolytic enzymes (see later), we have observed and studied to some extent certain other metabolic products. These include organic acids, polysaccharides, pigments and sterols.

Identification of oxalic and succinic acids as products of fermentation. It was noted (Tables 8 and 9) that P. palustris produced a pH of about 2 in shake culture within a week, hence this organism was used in studies to determine what acids were formed. The growth medium used consisted of the basal medium (Table 1), glutamic acid (0.012% total nitrogen), thiamine (1 mg. per liter), adenine (13.7 mg. per liter), and glucose (0.5%); pH 5.5 after sterlization. The medium was added in 1-liter amounts to 2-quart bottles. Incubation was at 28° C. for 1 week with forced aeration (2 liters per minute). After incubation the mycelium was separated from the culture fluid by filtration, and the liquid used for the identification of acid. The chemical procedure was, briefly, to extract the culture fluid with ether in a continuous extractor, evaporate the ether and recrystallize. Elementary analysis was carried out on the resulting white crystals and melting points and neutralization equivalents determined. The acid appeared to be oxalic. The p-toluidide derivative was prepared; its melting point (269.5°C.) checked closely with a known sample (269.3°C.) (The literature gives 268°C. as the melting point of this material). It was concluded that the acid was oxalic acid. No quantitative studies of its production have yet been carried out. With Fomes annosus in the medium described above, succinic acid was identified by preparing silver succinate.

Fungal polysaccharides. Several of our cultures of wood rots in shake culture in synthetic media produce large amounts of "slime". For F. meliae and P. abietinus the material appears to be a polysaccharide.

Pigments. Pigment production by several of the wood-rotting fungi has been noted in various synthetic media in shake culture, and has included yellow, orange, red and brown coloration in the medium and/or mycelium.

An orange, water soluble pigment was produced by Lenzites trabea in various synthetic media. Increased concentrations of certain of the trace elements (iron, 0.1 - 0.15 mg./l.; zinc, 0.14 - 0.21 mg./l.; molybdenum, 0.02 - 0.03 mg./l.; and boron 0.02 - 0.03 mg./l.) caused intensification of the pigment. In connection with the development of an optimal medium for L. trabea (see above), it was found that pigment was not produced when the concentration of glucose was 14% or more, regardless of other constituents. Maximum pigment formation took place in 2 - 4% glucose. That lack of pigment was not due to increased amounts of trace elements in the sugar, was shown by purifying the glucose by solvent extraction with dithiozone in carbon tetrachloride and 8 - hydroxyquinoline in chloroform. The use of this purified glucose still showed no pigment produced above a 14% concentration. When starch (up to 20% concentration) was substituted for glucose, pigment was still produced, suggesting that the mechanism of inhibition of pigment by glucose probably was an osmotic phenomenon.

With Lentinus tigrinus, a yellow-red pigment was produced in synthetic media. Pigmentation varied with the pH of the media, no pigment being produced (or at least apparent) below pH 4. At pH 5.5 the mycelial pellets were red and the solution yellow; at pH values above this both the pellets and solution were yellow. The pigment in this case acted to some extent as an indicator.

Three organisms - *L. striata*, *H. sollei* and *F. pini* produced intense orange pigmentation in the basal medium with glutamic acid. The pigment of *L. striata* was identified as a carotinoid, probably xanthophyll, by chemical tests and absorption spectra. *P. luteofibrata* and *M. lacrymans* in synthetic media produced a yellow pigment which turned red when exposed to light.

Preliminary results with chromatography indicated that this technique might be useful in the study of fungal pigments.

*Sterols*. Preliminary screening studies indicate that some of the wood rots form sterols in malt extract media, and smaller amounts in synthetic media.

## STUDIES ON CELLULOLYTIC ENZYMES

The wood-rotting Basidiomycetes are virtually the only organisms which can act on cellulose combined with lignin (as in wood), although many other types of microbes, as well as the wood rots, can act on more or less "pure" cellulose. It was therefore of great fundamental interest to investigate the production and characteristics of the cellulase(s) of these fungi.

*Methods for determining activity*.

Several methods were tried out to screen organisms for cellulolytic activity and/or assay cellulase. For convenience, these methods usually employed a cellulose substrate other than wood. The methods depended upon: A. Visual observation, and B. Chemical determination of end products.

A. Methods dependent upon visual observation for estimation of cellulase activity.

1. Growth of the fungi on cellulose agar plates. - - Medium: agar containing known nutrients, with added ball-milled filter paper or wood meal to produce opaqueness. Organisms streaked on surface and plates incubated; cellulase production supposed to produce zone of clearing around the line of growth. Composition of medium and concentration of cellulose rather critical. Best results with base layer of clear agar hardened in plate, then thin layer of cellulose agar on top. Slow growth of organisms and lack of clearing in many cases made this method impractical.

2. Growth in cellophane bag. - - Because the organisms attack cellophane (regenerated cellulose) their activity can be estimated from the time required for the bag to break. Bags made of cellophane tubing, and containing culture fluid, were suspended in more culture fluid inside of flasks. The fluid inside of the bag was inoculated with the organism. Cellulase breaks bag in a few days, but method is cumbersome and not very quantitative. Refinements might make the method useable, but probably not practical. Carboxymethyl cellulose films also were made fragile, but not cellulose acetate.

3. Growth in shake cultures containing finely-divided cellulose. - - Because of difficulty of separating cellulose and mycelium, the estimation of breakdown is not readily done. Breakdown of wood can be estimated by

the dye-absorbtion method (see later), assuming that the method measures cellulose. However, the dye-absorbtion method is not very suitable for the rapid determination of relative cellulase activity.

4. Growth on sheets of paper. - - Various kinds of paper sheets were placed on the surface of nutrient agar, inoculated and incubated. Weakening of some types of paper quite marked, but method not considered as good as some others.

5. The cellulase assay tube (drop-weight method). This apparatus can be used either for a growing liquid culture or for a filtrate. It consists of a glass tube open at both ends, smaller at one end than the other, and with both ends slightly flared. The tube fits into the neck of our standard shake flask (250-ml. Erlenmeyer). The lower end of the tube dips below the surface of the liquid (culture or filtrate) in the flask. This lower end has a narrow strip of cellophane across it; several stainless steel balls rest on the cellophane strip. (The cellophane is Dupont #450 P T, cut into strips 0.33 cm. wide. Five stainless steel balls (ball bearing) were used; they were 3/8" diameter, and each weighed 5.26gm.). Production or presence of cellulase in the liquid weakens the strip, allowing the balls to fall to the bottom of the flask. The time required for the strip to weaken sufficiently for the balls to fall is a measure of cellulase concentration or activity. Average cellulase activity allows the weights to drop in 72 to 96 hours. Unconcentrated cellulase.

(See Figure 6 below, for the construction and assembly of the apparatus). This method has been found very useful for screening of liquid cultures and/or culture filtrates for cellulolytic activity.

6. Dye-absorption method. A practical problem of some concern has been that of determining, in the presence of fungal mycelium, the amount of cellulose breakdown in sawdust in submerged culture. It has not been possible to separate the fine sawdust from the pellets of mycelium for this purpose, hence chemical methods of determining breakdown have not proved feasible. We have developed a selective dye-absorbtion method for this purpose, which appears to give consistent results. While we do not have incontrovertible proof that the method measures the disappearance of cellulose only, it appears probable that this is the case. Certainly the method measures the removal of some substance(s) from wood which has undergone fungal action in aerated culture, and controls on pure cellulose and on biologically-degraded wood of known lignin content strongly suggests that it is cellulose which the method determines. The method has not yet been investigated sufficiently to guarantee its reliability, but the technique and results are herewith presented tentatively. So far, only pine sawdust has been used.

In principle, the finely-divided wood, which has been subjected to the action of fungi, is stained in the presence of the fungal mycelium. The cellulose and

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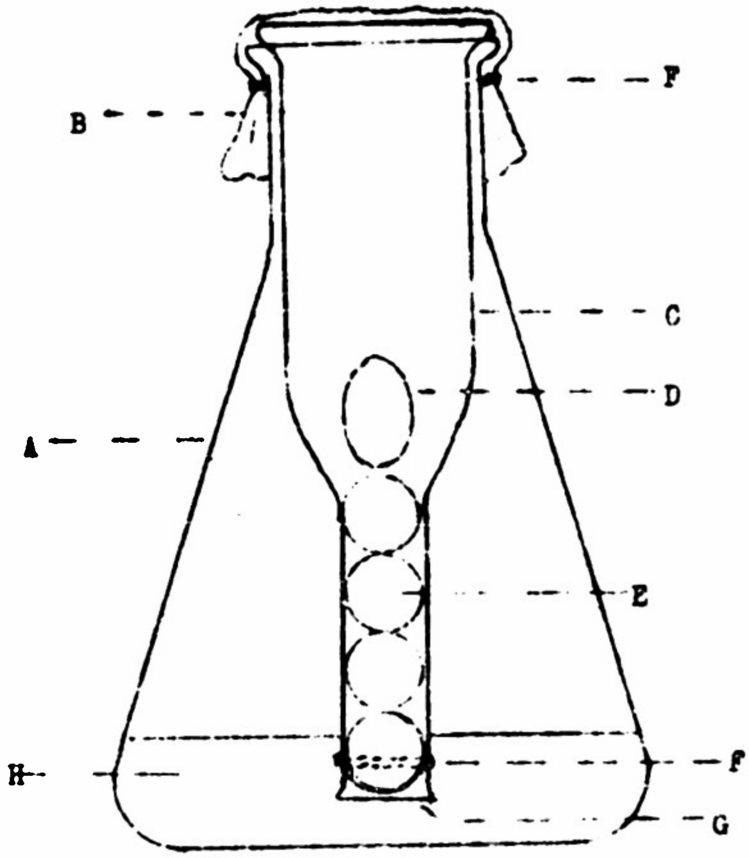


Vertical Section


Botton View

A 250-ml Erlenmeyer flask

B cellophane cap

C cellulase assay tube

D inoculation port

E stainless steel balls

F rubber collar

G cellophane strip

H culture medium or filtrate

FIGURE 6. CELLULASE A SAY TUBE ASSEMBLY

probably the lignin take the dye, and the mycelium also stains to some extent. The first de-staining (step 4, below) apparently removes most or all of the dye from the lignin and mycelium, but not from the cellulose. The second de-staining (steps 6-8, below) removes the dye from the cellulose, the amount of dye present and removed presumably being proportional to the amount of cellulose in the cellulose-lignin complex. The amount of dye removed by the second de-staining is quantitated in a spectrophotometer and, by comparison with a standard dye curve prepared using unfermented wood meal, the amount of cellulose breakdown is obtained. The details of procedure follow.

The wood meal (80 mesh) and mycelium from a shake culture (or other submerged culture) are collected on an asbestos mat in a Gooch crucible or in an alundum filtering crucible. The material is then treated as follows:

<u>Staining</u>

1. Moisten with 95% ethyl alcohol, then suck dry with vacuum.
2. Stain for 5 minutes with the following solution, then suck dry:

| | |
|---|---|
| malachite green | 0.5 gm. |
| distilled water | 150 ml. |
| ethyl alcohol, 95% | 50 ml. |

3. With suction, wash 20 ml. of distilled water slowly through the crucible.
4. With suction, drip slowly through the crucible 20 ml. of acid alcohol (1 part concentrated HCl plus 99 parts absolute ethyl alcohol) until

the filtrate is colorless.

5. Air-dry for 4 hours, or for 20 minutes at 100°C.

De-staining

6. Place the crucible and its dried contents in a 50 ml. beaker, and carefully pipette into the crucible without disturbing the mat of wood meal and mycelium, 10 ml. of the following solution:

| | |
|---|---|
| absolute ethyl alcohol | 50 ml. |
| methyl alcohol, 95% | 45 ml. |
| concentrated HCl | 5 ml. |

7. After 30 minutes the crucible is removed from the beaker, placed on a stainless steel mesh over the beaker, and allowed to drain into the beaker.

8. An additional 5 ml. portion of the above solution (6) is allowed to drain through the crucible, and is collected in the beaker.

9. Another 5 ml. portion of the same solution is used to wash any dye from the crucible and wire mesh.

10. The volume of the liquid collected in the beaker is brought up to 20 ml. with more of the solution.

11. An aliquot of the 20 ml. volume of de-staining solution, containing the dye eluted from the cellulose, is quantitated for dye concentration in a spectrophotometer.

The concentration of dye, compared with a standard prepared using unfermented wood, is a measure of the amount of cellulose broken down.

B. Method depending upon chemical determination of end-products of cellulase activity.

1. This depends upon the reducing characteristics of products of depolymerized cellulose, i.e., sugars, etc. As cellulose is

enzymatically attacked, the hydrolytic action is one of splitting the 1, 4-B-glycosidic linkage, making available a reducing group at the "1" position. The reducing capacity of the substrate is thus increased, such increase being in relationship to the number of linkages split. Metabolic fluid from cultures of wood rots is buffered and incubated, with a cellulose substrate, under toluol. Reducing substances which accumulate result from the action of the cellulase on the cellulose. These substances are determined by the Folin-Wu method for glucose, which depends upon the reduction of copper and of molybdenum. The intensity of blue color of the latter is proportional to the amount of glucose (or other reducing substance) in the test fluid. Spectrophotometrically, quantities of reducing substances (expressed as glucose) as low as 0.012 mg. per ml. can be determined. This is our preferred method for quantitative studies of cellulase activity, although it is not good as a rapid screening test.

Production of cellulase(s).

The culture fluid of several wood-rotting fungi was shown to have cellulolytic activity, indicating the presence of extracellular cellulase(s). Production (or activity) of the enzyme(s) was best when the organisms were grown in casein hydrolysate medium with several added nutilites. Qualitatively, the enzyme was demonstrated by various methods (chiefly the cellulase assay tube) in the culture fluid of the following brown rots: P. palustris, L. striata, L. trabea, D. quercina, P. rubescens, P. subroseus, T. serialis, P. immitus, P. spraguei, P. schweinitzii and P. nigra, and of the following white rots: P. anceps, P. versicolor, F. fomentarius and F. pini. P. palustris and L. trabea have been further studied in some detail, and will be used to illustrate methods and results.

Culture medium. The semi-synthetic medium developed to give a good production (activity) of cellulase, using P. palustris or L. trabea in shake culture, is shown in Table 22.

TABLE 22

Semi-synthetic medium for cellulase production with P. palustris or L. trabea in shake culture.

| | |
|---|---|
| Casein hydrolysate | 120 mg./l. nitrogen |
| $KH_2 PO_4$ | 1.5 g./l. |
| $Mg SO_4 \cdot 7H_2O$ | 0.5 g./l. |
| Adenine | 13 mg./l. |
| Guanine | 13 mg./l. |
| Uracil | 13 mg./l. |
| Thiamin HCl | 1 mg./l. |
| Glucose | 3 g./l. |
| Trace elements - same as in Table 1. | |
| Initial pH 5.0 | |

Studies with cellulose as an assay substrate. P. palustris was grown in the semi-synthetic medium (Table 22) in 1000-ml. volumes in 2-quart bottles with forced aeration for 5-10 days at 28°C. The culture was filtered through glass wool to remove gross particles, then through sintered-glass bacteriological filters to obtain a cell-free filtrate. Equal volumes (5 ml.) of the filtrate and 0.07 M phosphate buffer (pH 5.5) were put into a 50-ml. test tube containing 100 mg. of ball-milled filter paper. With a few drops of toluol as an antiseptic, the mixture was incubated at 28°C. for 4 days to 2 weeks. The mixture was then filtered, and the filtrate assayed for cellulase activity by determining the glucose in the filtrate before and after incubation, according to the Folin-Wu method given above.

Numerous tests were made varying the time of incubation of the original culture; relative amounts of assay solutions, buffer and substrate; temperature and time of assay incubation, etc. In no case was more than 0.03 mg./ml. of glucose found in the filtrate after incubation and assay. It was concluded either that cellulase was present in the filtrates in very small amounts and hence did not form much glucose from the filter paper, or that the conditions of assay were not correct to detect the products of cellulase activity. Since the amount of cellulase apparently could not be increased, as shown by the above method of assay, further work was directed towards modification of the assay procedure. It was thought that a more "reactive" cellulose might be more easily degraded, and hence give larger amounts of glucose for assay. The use of a modified cellulose as an assay substrate was found to give much better results (see below).

Studies with "reactive" cellulose as an assay substrate: The "reactive" cellulose was filter paper swelled with phosphoric acid. This was prepared as follows: Dried, ball-milled filter paper was placed in a beaker, and cold phosphoric acid (85%) sufficient to wet the paper was added. This was done in a salt water-ice bath at $0^{o}C$. After 30 minutes cold water was added to stop the reaction. The paper was washed with water several times on a Buchner funnel, then with dilute sodium hydroxide. After further washing to neutrality the preparation was dried, weighed and suspended in water to give a 2% concentration of the treated cellulose. This reactive cellulose was used in place of ball-milled filter paper in the assay procedure. The assay method was also changed in several other particulars.

P. palustris cell-free filtrates were prepared as indicated in the preceding section, with incubation for five days. For the assay, equal quantities (3 ml.) of filtrate, Mac Ilvaine's buffer (pH 4.4) and distilled water were mixed with 1 ml. of the 2% suspension of the phosphoric acid-treated cellulose. The mixture was incubated under toluol at 50°C. for 18 hours, then the glucose determined as before. Under these new conditions of assay with the reactive cellulose, 0.12 mg./ml. of glucose were present in the filtrate after incubation, as compared with 0.03 mg./ml. when untreated filter paper was used as the assay substrate. Apparently the modified cellulose is broken down to a greater extent than "natural" cellulose, giving larger glucose values which are analytically useful.

The above figures are illustrative of the data finally obtained after modifications were developed. Note the time (18 hrs.), temperature (50°C.) and pH (4.4) which were optimum for cellulase activity on phosphoric acid - treated cellulose. Filter paper treated with phosphoric acid for less than 30 minutes was found to give lower glucose values than when treated for 30 minutes. Mycelial extracts were found to contain no cellulase; apparently it is a true extracellular enzyme. The modified cellulose gave such good results that a soluble cellulose derivative was next tried.

Studies with a cellulose derivative as an assay substrate. Carboxymethyl cellulose (CMC*), a soluble substance, was tried as an assay substrate. This had been shown by other workers (Reese et al, 1950) to be easily hydrolyzed by cellulolytic enzymes of other organisms.

The filtrate from a 5-day old culture of P. palustris was prepared

* Sample designated CMC 50 T, with a degree of substitution of 0.52.

3

as previously described, and assayed as in the proceding section except that a 2% solution of CMC in water was substituted for the 2% phosphoric acid-treated filter paper. After incubation a glucose value of 0.61 mg./ml. was found, compared with 0.12 mg./ml. when phosphoric acid-treated cellulose was used. Thus the cellulase can attack soluble cellulose more readily than insoluble forms. With this assay substrate, glucose values up to 0.75 mg./ml. have been obtained with *P. palustris*, depending upon culture age, etc.

Studies of this assay technique with *P. palustris* filtrates have shown that a 0.5% solution of CMC gives the same results as 2%, that a variation in assay pH between 3.4 and 5.0 is of little significance, and that an assay temperature of 60°C. results in markedly more glucose than 50°C. Also, with this new technique the assay may be run in 3 hours instead of 18 if desired; for example, in one test 0.53 mg./ml, of glucose were formed at 50°C. in 3 hours.

Culture filtrates of both *P. palustris* and *L. trabea* showed maximum cellulase activity when the cultures were 3-4 weeks old.

*Concentration of cellulase.* It was of interest to determine whether the enzyme could be concentrated or removed from the culture fluid by common protein precipitants. Those tried were saturated ammonium sulfate, ethyl alcohol, acetone, and mixtures of alcohol and acetone. It was found that the precipitants had to be pre-cooled to -15°C. and the filtrate to 0°C., and the precipitation carried out at 0°C. Various quantities of the precipitants were mixed with various volumes of the cell-free culture fluid of *P. palustris*, and the precipitates taken up in MacIlvaine's buffer (pH 4.4), in which the precipitates were nearly 100% soluble. Assay of cellulase activity was made as before, using 4 ml. of buffer - precipitate mixture to 1 ml. of 2% CMC, and incubating for 18 hours at 50°C.

The results with P. palustris showed that the enzyme could be precipitated by any of the precipitants used, with acetone, and alcohol-acetone 1:1, being by far the best. However, in the few experiments carried out, it was not possible to concentrate the enzyme more than 3-fold by this procedure, due to "inactivation" or some other unknown reason. For example, the precipitate from 150 ml. of acetone plus 50 ml. of culture fluid was taken up in 10 ml. of buffer and assayed. Glucose was only 0.31 mg./ml. as compared to 0.12 mg./ml. for the unconcentrated (unprecipitated) filtrate.

Incidentally, it has been found that both culture filtrates and concentrated enzyme can be preserved with merthiolate (0.01%) for at least 3 weeks (end of test) without loss of activity.

Use of the above protein precipitants with culture filtrates of L. trabea gave a "concentrate" which, when assayed against filter paper, showed only a trace of activity. When assayed against carboxymethyl cellulose, 0.31 mg./ml. of glucose were produced, the same as with P. palustris.

Preliminary studies were made with L. trabea using ion exchange resins for purification of cellulase in culture filtrates. The resins which gave positive results were Ionac* resins # A-293-M and # C-200. These were placed in chromatographic columns, and the culture filtrate passed over them. When used singly no activity of the filtrate resulted, but when passed first over # A-293-M** and then # C-200***, followed by

* American Cyanamid Co., N.Y.
**Picks up anions and replaces them with hydroxyl ions.
***Pick up cations and replaces them with hydroxyl ions.

concentration (4x) by vacuum distillation, the cellulase activity of the resulting fluid was about 5 times that of the original filtrate. (Vacuum distillation alone did not increase the activity of a filtrate, due perhaps to "inactivation" of the enzyme by salts and acids present, plus heat. The object of the ion exchange was to remove these "interferring" substances. The distillation was carried out in glass, evacuated by a water pump attached to the receiver. The receiver was placed in an ice bath, and the distilling flask maintained at 45°C.) For example, one original cell-free filtrate gave 0.11 mg./ml. of glucose against filter paper; after passage through both resins the value was 0.12 mg./ml. and after concentration of the latter to 1/4 its volume the value was 0.51 mg./ml. of glucose.

It is to be noted that the cellulase of *L. trabea*, when assayed against filter paper, gives much higher values than the cellulase of *P. palustris* against filter paper.

The above data show that wood-rotting fungi produce extracellular cellulolytic enzyme(s). Whether there is one enzyme or a mixture is not clear. The small amount of enzyme action on filter-paper cellulose may be interpreted either as there being only a small amount of "true" cellulase present, or as the long chain-length of "pure" cellulose being difficult to break. Another possibility is the absorption of a relatively small amount of enzyme on so much cellulose substrate that the enzyme - substrate ratio is too small for much action. The increasingly greater amounts of breakdown of phosphoric acid-treated filter paper and of carboxymethyl cellulose may be a reflection of greater amounts of a different "cellulase" capable of acting on modified, simpler celluloses, or may merely reflect the greater ease of hydrolysis by a "true" cellulase of short-chain and soluble celluloses. Reese et al (1950) have restricted the term cellulase to the enzyme acting on native cellulose; the enzyme acting on carboxymethyl cellulose is carboxymethyl cellulase.

Effect of medium constituents on cellulase production. All of the above studies of cellulase were based on growth in a semi-synthetic medium (Table 22), with casein hydrolysate as the source of nitrogen, and glucose as the carbon source. Using L. trabea, studies were made of the effect of the type of carbon source on cellulase production and, with a synthetic medium, the effect of different nitrogen compounds. All assays were against filter paper, and carried out as above under "Studies using "reactive" cellulose as an assay substrate."

Data on various carbohydrates substituted for glucose in the semi-synthetic medium showed no differences in cellulase production among glucose, sucrose, maltose, mannitol, cellobiose, starch, lactose, galactose, and carboxymethyl cellulose.

A mixture of pure amino acids, in types and concentrations approximating those in the casein hydrolysate, was substituted for the latter in the semi-synthetic medium. The same amount of cellulase (as shown by glucose values) was produced in this chemically-defined medium. A series of tests were then made in which one amino acid at a time was omitted from the mixture, and cellulase production determined. The results showed no difference in cellulase production between the complete medium and the media in which any one amino acid was missing. Next, two amino acids at one time, in all possible combinations, were omitted from the complete mixture. It was found that the elimination of valine, aspartic acid and glutamic acid together resulted in a large decrease in cellulase activity, e.g.: complete amino acid medium, 0.54 mg./ml. of glucose; complete medium minus valine, aspartic acid and glutamic acid, 0.19 mg./ml.

With the above information as a basis, valine, aspartic acid and glutamic acid were substituted for the complete amino acid mixture, and when the filtrate was assayed a glucose value of 0.46 mg./ml. was obtained. This compares favorably with 0.54 mg./ml. obtained in the complete amino acid medium. The optimum concentrations of glutamic acid, valine and aspartic acid were, respectively, 0.0096, 0.0032 and 0.0032% nitrogen. Thus a purely synthetic medium for cellulase production by *L. trabea* at least, can be made by substituting the above amino acids for the casein hydrolysate of the semi-synthetic medium in Table 22.

# ACTION OF WOOD-ROTTING FUNGI ON WOOD

Preliminary study of the action of various of our fungi on finely - divided wood in liquid culture showed that the wood apparently was digested. The organisms were grown in culture with forced aeration, using casein hydrolysate plus vitamins. The wood was pine, added in the form of wood meal (fine sawdust of 80 mesh). With certain of the fungi the woodmeal disappeared within a week. Stained sections of the pellets of growth showed particles of the wood entangled in the mycelium inside of the pellet. Apparently as the pellets grew they occluded wood particles in a more or less regular fashion. It was not certain from these experiments whether the disappearance of the woodmeal was due entirely to physical entrapment by the mycelium or whether some particles were actually digested in the medium by the fungal enzymes.

The dye - absorption method (# A, 6, above, in section on Studies on Cellulolytic Enzymes) was developed for the purpose of determining the degree of breakdown, if any, of finely - divided wood. Using this method the following organisms were tested for their action on 80-mesh pine wood meal added to shake cultures: brown rots - - *P. incrassata*, *P. microspora*, *P. palustris*, *L. lepideus*, *L. trabea*; white rots - - *P. tulipiferus*, *P. anceps*, *F. annosus*, *L. tigrinus*. The culture medium consisted of the basal medium (Table 1) plus glucose 0.01%; thiamine, 2 mg. per liter; sodium glutamate to 0.012% total nitrogen; pine wood meal, 0.01% (air dry basis). Incubation was for 14 days at 28°C., with triplicate flasks. After incubation, the mixture of mycelial pellets and wood meal was removed on a Gooch crucible, and the staining method described above was carried out.

Assuming that the staining method determines cellulose, the following centages of removal of cellulose from the wood were obtained:

| | |
|---|---|
| *P. incrassata* | 27% |
| *P. monticola* | 31% |
| *P. palustris* | 40% |
| *L. lepideus* | 35% |
| *L. trabea* | 42% |
| *P. tulipiferus* | 38% |
| *P. anceps* | 22% |
| *F. annosus* | 16% |
| *L. tigrinus* | 31% |

Uninoculated controls run under the same conditions showed an average of 4.3% of the cellulose (?) removed from the wood. Obviously the different organisms vary in their ability to remove "cellulose" from the cellulose-lignin complex. The white rots, which are known to attack both cellulose and lignin, removed about the same amount of "cellulose" from the wood as did the brown rots which attack only cellulose. These studies were not carried further.

A few studies were made on the degradation of bark by *P. palustris* in aerated liquid culture. It was demonstrated that the cellulose (circa 20%) of spruce bark is attacked by wood-rotting fungi, in an effort to meet their nutritional needs, with a production of certain desirable end-products, including reducing substances (expressed as glucose). The production of alcohol by the yeast, *Saccharomyces cerevisiae*, from bark treated by the brown rot, *Polyporus palustris*, was also established. Spruce bark cellulose degradation was also effected by the use of a cell-free filtrate of a *Polyporus palustris* liquid culture. The production of reducing substances (expressed as glucose) in this case equalled that of bark attacked directly by the organism. Subsequent alcohol production by yeast from bark treated with cell-free filtrates of Polyporus palustris was also induced. Also, using a sterilised mixture of bark in water, inoculated with P. palustris, there was a definite decrease in reducing substances noted in the liquid immediately following growth of the organism. An increase in reducing substances took place after incubation at 45°C., the optimum temperature for enzymatic activity (lethal temperature for the organism). Therefore it may be inferred that the best yield of reducing substances will be obtained by the proper use of a cell-free filtrate, as there is not utilisation of material by any organism.

In addition to bark, we have also found that reducing sugars can be formed from corn cobs as the cellulose substrate. It was not possible to continue this work, but it offers important practical possibilities for the utilisation of waste cellulose.

## ACTIVE MYCELIAL EXTRACTS FROM THE WOOD ROTS

Cell-free, enzymatically-active mycelial extracts would be useful for numerous enzyme studies. Preliminary attempts to obtain such extracts were inconclusive when mycelial pellets were blended in a mortar with sand and buffer at room temperature, and the filtrate tested in the Warburg manometer with sodium pyruvate. Absence of oxygen uptake indicated either no carboxylase enzyme in the mycelium, or inactivation of this enzyme by the method of preparation. Further studies, using P. palustris, have yielded a cell-free extract which showed carboxylase activity. Presumably the same technique could be used for obtaining other endo-ensymes.

Mycelial pellets were obtained by growing the organism under forced aeration in two-quart bottles containing 2% malt extract. Incubation was at 28°C. for four days. The pellets were then separated from the medium by pressing through cheesecloth, and the mycelium lyophilized and pulverised. 0.5 grams of the pulverized mycelium were transferred to a 50-ml. centrifuge tube, 10 ml. of phosphate buffer (pH 5.0) added, and the tube shaken for one minute. The suspension was then dentrifuged for three minutes at 2000 rpm, and the supernatant decanted for activity studies. The carboxylase activity of the extract was determined by measuring the rate of $CO_2$ evolution from sodium pyruvate with standard Warburg manometer procedures, at 28°C.

Preliminary studies of the influence of pH on carboxylase activity showed pH5 to be better than pH6. At the former pH, 154.3 microliters of $CO_2$ per hour were evolved; at the latter pH, 130.8.

To determine whether the cell-free extract contained cocarboxylase as well as carboxylase, runs were made with sodium pyruvate in a concentration of 5 mg./ml. of extract, with the "standard" extract, standard extract plus 2 micrograms cocarboxylase (pure ensyme) per ml., and standard extract plus 5 micrograms cocarboxylase per ml. Microliters of $CO_2$ per hour were, respectively, 130.8, 128.6 and 129.8, showing that cocarboxylase was present in the extracted mycelium, and further addition of it to the extract had no appreciable effect.

The effect of the enzyme-substrate concentration on $CO_2$ evolution was studied at two concentrations of substrate and four concentrations of enzyme, at pH5.0, with no added cocarboxylase. Table 23 shows the cumulative results at 10-minute intervals, of $CO_2$ evolution expressed as microliters per hour. The results show that the breakdown of sodium pyruvate by carboxylase is influenced by the concentration of ensyme and substrate.

TABLE 2

Carboxylase activity of cell-free extract of P. palustris. Effect of enzyme-substrate concentration with sodium pyruvate, at pH5.0, 28°C . Activity expressed as microliters $CO_2$ per hour, at 10-minute intervals. S= "standard" extract concentration.

Substrate: sodium pyruvate
Concentration: 1 mg./ml. of extract

µl. $CO_2$/hour

| Time in minutes | Concentration of enzyme extract: S | 0.5 S | 0.25 S | 0.125 S |
|---|---|---|---|---|
| 10 | 28.1 | 21.3 | 7.95 | 8.58 |
| 20 | 56.2 | 37.4 | 17.5 | 15.5 |
| 30 | 67.0 | 42.7 | 20.7 | 15.5 |
| 40 | 82.6 | 55.2 | 27.0 | 18.9 |
| 50 | 98.2 | 67.6 | 35.0 | 27.4 |
| 60 | 110.6 | 74.7 | 39.7 | 30.9 |
| 70 | 116.8 | 83.6 | 44.5 | 32.6 |
| 80 | 123.1 | 88.9 | 46.1 | 34.3 |
| 90 | 127.8 | 94.3 | 50.9 | 37.8 |
| 100 | 132.4 | 97.8 | 52.5 | 37.8 |
| 110 | 133.9 | 103.2 | 57.2 | 41.2 |
| 120 | 137.1 | 108.5 | 62.0 | 46.4 |

Substrate: sodium pyruvate
Concentration: 5 mg./ml. of extract

µl. $CO_2$/hour

| Time in minutes | Concentration of enzyme extract: S | 0.5 S | 0.25 S | 0.125 S |
|---|---|---|---|---|
| 10 | 34.6 | 18.6 | 9.34 | 8.91 |
| 20 | 69.2 | 40.6 | 20.2 | 13.4 |
| 30 | 85.1 | 48.1 | 23.3 | 16.3 |
| 40 | 109.6 | 64.5 | 31.1[illegible] | 22.3 |
| 50 | 126.8 | 80.6 | 40.4 | 29.7 |
| 60 | 154.3 | 92.9 | 46.7 | 34.2 |
| 70 | 168.6 | 100.7 | 49.8 | 35.6 |
| 80 | 184.5 | 111.6 | 54.5 | 38.6 |
| 90 | 198.9 | 120.8 | 59.1 | 43.1 |
| 100 | | 130.2 | 62.2 | 44.6 |
| 110 | | 142.6 | 70.0 | 49.0 |
| 120 | | 151.9 | 76.2 | 53.5 |

## CONCLUSIONS

The nutrition and certain aspects of the physiology of forty-three brown rots and white rots have been studied in shake cultures at 28°C. The following general conclusions may be drawn from these investigations.

1. These fungi generally will grow in a variety of non-synthetic media such as malt extract, corn steep liquor, ethyl stillage, soy bean extract, gluten, peptone and casein hydrolysate.

2. Most of the wood rots will grow in continued subculture in a basal synthetic medium of glucose, inorganic salts, thiamin and organic or inorganic nitrogen. A few have special nutritional requirements.

3. Utilizable nitrogen compounds included any single amino acid (L-form only), ammonium sulfate, ammonium carbonate, urea, and ammonium nitrate. No growth was obtained in potassium nitrate or potassium nitrite. Growth took place in ammonium chloride only in the presence of traces of succinic acid.

4. Most of the species studied require only thiamin as an added vitamin in the culture medium. Some species require the whole thiamin molecule; others can utilize one or the other component of the molecule separately, or both together. Two organisms required no vitamins; two others had vitamin requirements in addition to thiamin.

5. For most of the species studied, biotin could be substituted for thiamin. There was little or no synthesis of thiamin by cultures growing with biotin.

6. Other vitamins and purine or pyrimidine bases often stimulated growth, but are not essential.

7. The optimum pH for most of the organisms studied was about 5.0- 5.5. Some species have an optimum somewhat lower than this.

8. A temperature of 28°C. is satisfactory for the great majority of the wood rots, although a few species have an optimum somewhat lower.

9. Most of our organisms produced an acid reaction in various types of culture media. pH values as low as 2 - 3 were not uncommon. Oxalic acid was identified as one acid produced.

10. Maximum growth of the organisms in shake culture was attained in an average of 14 days.

11. An Eh of $+$ 400 to $+$ 500 m.v. was satisfactory for the growth of all but one of the fungi studied. This organism required a more reduced Eh of about $+$ 300 m.v.

12. Amino acids could not be utilized as the sole source of carbon by any of the organisms.

13. Synthetic media more nearly optimal for growth than the basal medium were developed by increasing the concentrations of glucose, nitrogen and potassium phosphate. Increases in growth up to 37-fold were obtained with some organisms in the new media.

14. A variety of carbon compounds could be utilized in place of glucose. These include fructose, galactose, glycerol, lactic acid, lactose, maltose, pyruvic acid, succinic acid, sucrose, mannose, dextrin, xylose, glycogen, arabinose, ribose, rhamnose, cellobiose and starch. Not utilized were acetic acid, citric acid, maleic acid and tartaric acid.

15. Mass culture of wood-rotting fungi in aerated liquid culture was found feasible.

16. In addition to acids, other products of fermentation and/or synthesis are pigments, polysaccharides and sterols.

17. The wood-rotting fungi produce in various media an extracellular cellulolytic enzyme. This enzyme can be separated from the culture medium by precipitation or by ion exchange resins and concentration <u>in vacuo</u>.

18. A cellulase assay tube (drop-weight method) was developed and found useful for screening fungi for cellulolytic activity.

19. A purely synthetic medium, containing three apparently essential amino acids, was developed in which cellulase production was as good as in a casein hydrolysate medium.

20. Active mycelial extracts, as determined by carboxylase activity, were obtained by lyophilizing and pulverizing the mycelium.

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(REVIEW OF LITERATURE A)


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